Author:
Kandage, Dhinushi

Title:
Modulating anti-tumour-specific CD8+ cytotoxic T lymphocyte responses

Modulating anti-tumour-specific CD8+ cytotoxic T lymphocyte responses

S Dhinushi Kandage
Student Number 1654463

Supervisor: Dr. David J Morgan
School of Cellular and Molecular Medicine

Start Date: 3rd October 2016

Abstract

The induction of CD8+ cytotoxic T lymphocytes (CTL) is vital in the immune response to tumour growth, and the presence of tumour-infiltrating CTL correlates with positive prognoses. Immunotherapies aim to either induce potent anti-tumour immune responses through vaccination, or to increase the numbers of tumour-specific CTL in patients and/or strengthen their effector functions against tumours. However, within the tumour microenvironment (TME) many immunosuppressive mechanisms operate to prevent cytotoxic activity amongst CD8+ tumour-infiltrating T lymphocytes (TIL). Previous studies have shown that loss of CTL function within the TME is associated with the influx of large numbers of FoxP3+CD4+ regulatory T cells expressing ectoenzymes CD39 and CD73 that catalyse stepwise adenosine production. Other studies also showed that there is an upregulation in the expression of coinhibitory receptors (CIRs) amongst CD8+ TIL compared with CTL. We hypothesised that production of immunosuppressive adenosine may also contribute to CTL effector function loss through upregulation of CIRs, thus promoting continued tumour growth. Studies detailed in this thesis were carried out in order to identify changes in the expression patterns of the CIRs PD1, TIM3, TIGIT and LAG3 and in CTL proliferation amongst tumour-specific CD8+ T cells in the presence of 5′-(N-Ethylcarboxamido*)* adenosine (NECA), an adenosine receptor agonist; and to test the ability of a nove*l* vaccine strategy using scaffold proteins called Self-Assembling peptide caGEs (SAGE) to deliver tumour-specific peptides to stimulate CD8+ T cell proliferation *in vivo.* The data show that NECA did not affect expression patterns of PD1, TIM3, TIGIT and LAG3 during CTL priming *in vitro*. However, we do show that SAGE constructs are capable of driving strong proliferative responses of CTLs *in vivo*. These findings provide further insight into the immunosuppressive mechanisms within the TME as well the immunotherapeutic potential of using novel SAGE-conjugated peptides to stimulate anti-tumour CTL responses *in vivo.*

Abstract word count: 300

Author’s Declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's *Regulations and Code of Practice for Research Degree Programmes* and that it has not been submitted for any other academic award. Except where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

_____ _____

Signature Date

Acknowledgements

First, I would like to thank my supervisor Dr. David Morgan for his help throughout my project and for giving me the opportunity to learn and participate in the research of his group.

I would also like to thank Dr. Andrew Herman for his guidance with flow cytometry. I owe thanks to Prof. Linda Wooldridge for her help and advice to help me progress through my project. Additionally, I would like to thank Holly Baum and Caroline Morris for their help and time.

I would like to thank Grace Edmunds, for the colossal amount of advice she provided me with and for making time in her busy schedule so often to help me with my work.

Huge thanks to everyone in the office: Hon Lam, Lorena Sueiro Ballesteros (also for her help with all things flow cytometry!), Lea Knezevic. Emily Milodowski, Ore Francis, Anya Lissina and Cath Lewis for the many entertaining hours and snacks in the office to brighten up even the dullest days.

Last but not least, I would like to express my loving thanks towards my parents, sister and auntie for pushing and supporting me in the pursuit of my interests. I would like to thank Oliver Crowther without whom I would not have achieved this Masters; I am eternally grateful for his endless support and motivation throughout which allowed me to complete this project, and for putting up with me.

Table of contents

Table of Figures

List of Tables

Abbreviations

A2AB:	Adenosine A2B receptor
A2AR:	Adenosine A2A receptor
ADA:	Adenosine Deaminase
AK:	Adenosine Kinase
AMP:	Adenosine monophosphate
APC:	Antigen-presenting cell
ATP:	Adenosine triphosphate
CIR:	Co-inhibitory Receptor
CTL:	Cytotoxic T lymphocyte
CTLA4:	Cytotoxic T-lymphocyte-associated protein 4
CTV:	Cell-Trace Violet
CXCL:	Chemokine ligand
DC:	Dendritic cell
DNA:	Deoxyribonucleic acid
ENT:	Equilibrative nucleoside transporters
EP:	Epitope Peptide
FMO:	Fluorescence Minus One
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
HA:	Haemagglutinin
IFN:	Interferon
IL:	Interleukin
IRAE:	Immune-related adverse events
LEA:	Leader Peptide
LAG3:	Lymphocyte activation-gene 3
mAbs:	Monoclonal antibodies
MACS:	Magnetic-activated cell sorting

MHC: Major histocompatibility complex

NECA: 5'-(N-Ethylcarboxamido) adenosine

NK: Natural killer

PAP: Prostate acid phosphatase

PD1: Programmed cell death-1 receptor

PFA: Paraformaldehyde

POL: Pol-loop peptide

PSR: Pulsed Splenocyte Reaction

SAGE: Self-assembling peptide cage

TCR: T cell receptor

TDLN: Tumour-draining lymph node

TIGIT: T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif

TIL: Tumour-infiltrating lymphocyte

TIM3: T-cell immunoglobulin and mucin-domain molecule 3

TME: Tumour microenvironment

TNF: Tumour necrosis factor

T_{reg}: Regulatory T cell

WT: Wild Type

Chapter 1 - Introduction

1.1 T Cell Lymphocytes

T lymphocytes play an essential role in the prevention and elimination of cancer by attacking malignant cells to clear these from the body. Immature thymocytes are generated from progenitor cells in the thymus. By undergoing positive selection, double positive cells (CD4+/CD8+) with the ability to react with major histocompatibility complex (MHC I) molecules are furthered to differentiate into single positive CD8+ T cells. Following positive selection, these cells continue through negative selection. Thymocytes that bind 'self' MHC peptides undergo apoptosis to prevent the accumulation of self-reactive T cells and provide central tolerance. Cells that did not undergo apoptosis leave the thymus and migrate to peripheral lymphoid tissues to mature (1).

Cytotoxic T lymphocytes (CTL) are primed when naïve CD8+ T cells encounter an antigen-presenting cell (APC) presenting specific tumour antigens via MHC Class I molecules to the T cell receptor (TCR). In combination with co-stimulation through B7.1 and B7.2 on the APC surface binding CD28 on the T cell surface, CD8+ T cells are activated to become CTLs and thus begin proliferating as well as producing effector cytokines, such as interleukin-2 (IL-2) and interferon-γ (IFN-γ) (1, 2). Following activation, some activated CTL differentiate into central memory T cells. These migrate to secondary lymphoid tissues where they are maintained with little or no function. Upon subsequent exposure to the same antigen, they become highly functional cells with the ability to proliferate rapidly and produce a variety of cytokines. Central memory T cells establish a faster and stronger immune response to re-exposure of the antigen, providing last-longing immunity (3, 4).

Dendritic cells (DC) which are considered professional APCs may also internalise and present exogenous, rather than endogenous, antigens via MHC I to the CD8+ T cell TCR. Other APCs have also been observed to cross-present, however it is most commonly observed with DCs (5). Cross-presentation is crucial during infections as APCs can elicit cytotoxic responses from CD8+ T cells without being affected themselves. Internalised peptides are processed by either the cytosolic or the vacuolar pathway. When entered into the cytosolic pathway, peptides are

degraded by the proteasome in the cell cytosol, whilst in the vacuolar pathway the processing occurs by proteolysis from lysosomes (6).

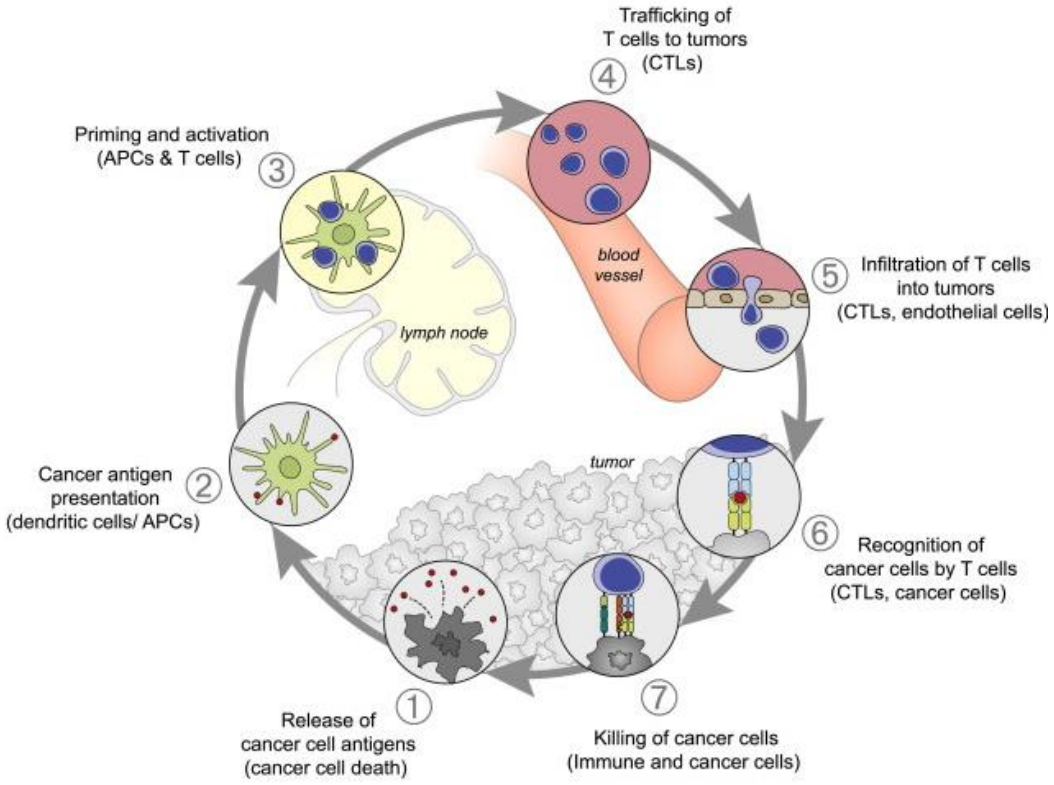

Figure 1.1 - T Cell Activation and Migration

Naïve CD8+ T cells are activated in secondary lymphoid tissues, such as the lymph node, and after presentation of tumour antigen by antigen-presenting cells (typically dendritic cells) in combination with costimulatory signals, they differentiate into CTL effector cells. Subsequently CTL enter circulation and, with the help of chemokines, travel to the tumour site to infiltrate its stroma. TCR on the T cells allows recognition of the target cells, triggering cytotoxic killing mechanisms. This leads to cell death of cancer cells, debris of which is in turn picked up by antigen-presenting cells that travel to nearby lymph nodes to present it. *(Adapted from Chen et al, 2013* (75))

Activated anti-tumour CTLs travel to the site of the tumour to infiltrate it and attempt to eradicate the cancer cells, as shown in Figure 1.1. CTLs eradicate target cells primarily through the release of cytolytic granules, but also via the Fas pathway. These granules contain perforin which forms pores in the target cell's membrane. Additionally, Tumour-necrosis factor and IFN-γ are of cytotoxic nature when produced by activated CTLs in the proximity of targets. Once activated, CD8+ T cells also upregulate the expression of Fas ligand on their surface; once Fas receptors on target cells are engaged by the ligand, the apoptotic pathway is triggered (7, 8).

However, despite the specific priming and activation of CTLs for anti-tumour activity, clearance of established tumours by the immune system is rare. Further, tumour-specific CTLs that show

effective tumour killing ability *in vitro* undertake no such activity in the tumour and are tumour-tolerant *in vivo* (9).

1.2 Immune Evasion by Tumours

Cancer is the abnormal and uncontrollable growth of cells due to DNA damage from environmental and/or genetic causes. This malignant growth generally results in tumours and has the potential to metastasise to other tissues via the blood or lymphatic system. In the present age, more than 360 000 new cancer cases occur in the UK yearly and 1 out of 2 people born after 1960 will develop a form of cancer (10). Chemotherapy, radiation therapy and sometimes surgery are the traditional methods of treatment for cancer and although these treatments have helped to reduce the number of cancer deaths by providing means of intervention, they are still far from ideal. In chemotherapy patients are administered medication, such as Tamoxifen or Imatinib, which target tumour proliferation. Unfortunately, these drugs also affect healthy cells with high cell division, such as hair cells, skin cells and cells of the gastrointestinal tract, resulting in unfavourable side effects such as nausea, vomiting, hair loss and fatigue (11, 12). Radiotherapy uses radiation to stop the proliferation of tumour cells. Due to the difficulty in targeting the radiation to only the tumour cells, healthy surrounding cells are damaged collaterally. This damage results in treatment-related side effects including headaches, immunocompromise, subsequent infections and fatigue (13, 14). The low specificity as well as the physical and mental toll on cancer patients highlights the need to develop more effective and non-invasive treatment options. Immunotherapy which is based on utilising the host's own immune system has been actively researched in recent years and provides a very appealing alternative.

As normal cells undergo the transformation into cancer cells, they can develop neo-antigens which the immune system may recognise and react against. But often tumours avoid eradication by the immune system through various means, such as local immune evasion, disrupting T cell signalling and prompting immune tolerance (15, 16). For example, the loss of MHC class I expression on tumour cells prevents their recognition by anti-tumour CTLs (17), and although this reduced expression makes the cancer cells favourable targets for natural killer (NK) cells, downregulation of NK molecules as well as reduced activity of NK cells can often be observed in cancer patients (18, 19).

Immunoediting refers to the process in which the anti-tumour immune response towards malignant cells not only prevents, but also inadvertently promotes tumour growth. It consists of three phases, which are illustrated in Figure 1.2; and it explains the underlying processes of tumour growth as well as evasion of immune responses (20, 21).

In the initial Elimination phase, immunosurveillance occurs during which innate effector cells such as natural killer cells recognise malignant cells and produce IFN-γ in response. This results in inhibition of further proliferation of the cancer cells and the subsequent the production of chemokines such as CXCL9 and CXCL10, recruiting DCs and other immune cells to the site. Subsequently also antigen-specific CD4+ T cells and CD8+ T cells arrive to eradicate the malignant cells. Tumour cells with reduced immunogenicity however can escape this process (20-22).

During the next phase, Equilibrium, cancer cells that avoided killing during immunosurveillance are selected for growth by the tumour. As immune cells recognise certain tumour neoantigens during immunosurveillance, this provides selection pressure for the tumour cells to not be recognised. This phase can last several years as different mutations may occur in the cells (22, 23).

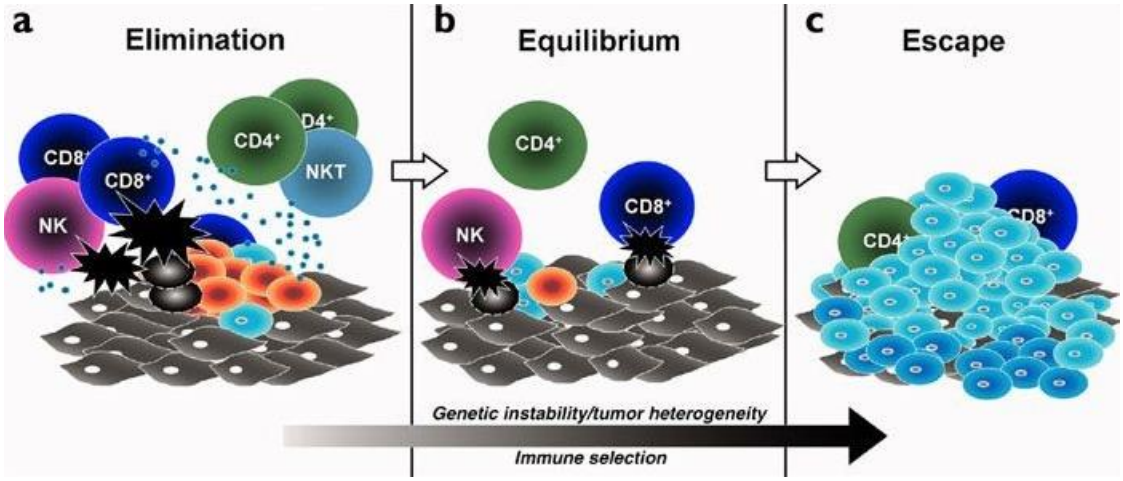

Figure 1.2 - The Phases of Immunoediting

Immunosurveillance detects cells in (a) and eradicates them in response. (b) shows the equilibrium stage, where the immune system inadvertently selects and supports mutated tumour cells that manage to not be eliminated. This process results in (c), where different variants of the tumour cells have generated and grown uncontrollably, undetectable by the immune system.
Blue represents different mutated tumour cells, red: tumour cells undergoing proliferation, grey: non-transformed neighbouring cells in the stroma, black flashes: cytotoxic activity against tumour cells and small blue circles are cytokines. (*Adapted from Dunn et al, 2002 (23)*)

Escape is the final phase of immunoediting. The selected-for cancer cells now continuously divide uncontrollably, whilst being undetected or unsusceptible to the host's immune system (20, 23).

1.3 Immunosuppression in the TME

The tumour microenvironment (TME) of solid tumours is highly immunosuppressive and contributes strongly to the lack of immune response against the tumour, and thus hinders its eradication. Many tumours continue to grow regardless of the presence of primed anti-tumour infiltrating T lymphocytes (TIL) within tumour stroma (24). Often the rapid growth of cancer cells exceeds the rate of angiogenesis, which can cause a lack of oxygen and nutrients to surrounding healthy tissues which, in turn, creates areas of severe hypoxia and necrosis of the surrounding tissues (25).

Contributing to this immunosuppressive environment are regulatory T (T_{reg}) cells, whose normal function is, amongst other things, to suppress autoreactive T cells to prevent autoimmunity. Tumours have been shown to express specific chemokines, such as CCL22, to attract T_{reg} cells to the tumour site (26). The support of tumour growth by the presence of T_{reg} cells is highlighted when T_{reg} cells were suppressed and clearance of tumours as well as increased efficacy of immunotherapies were observed (27, 28). Additionally, the large number of T_{reg} cells present in the TME contribute to high levels of anti-inflammatory molecules. The production of tumour necrosis factor (TNF) by other immune cells present also encourages growth of cancer cells (29).

Due to the cytotoxicity of CTLs, it is important to control their activity to prevent excessive cytotoxic action. This may be done via coinhibitory receptors (CIRs) which exist to regulate T cell activity. However, engagement of CIRs expressed on CTLs that are primed against tumour-specific antigens suppresses their anti-tumour functions and can cause immune tolerance towards the tumour (24). CIR expression is highest on T cells found in the TME, followed by cells in normal tissue. T cells in peripheral blood display the lowest levels of inhibitory molecules (24). This strongly indicates that the TME induces and maintains the expression of CIRs on CTLs and thus inhibits their effector functions.

In response to persistent activation in cases of chronic infection or uncontrolled tumour growth, T cell effector functions are reduced or lost (3, 30). This state is called T cell exhaustion. T cell dysfunction has sprung from the importance of regulating T cell activity to reduce excessive immunopathology (30). Exhaustion varies from T cell anergy in that anergic T cells enter this state due to lack of costimulatory signals during activation and thus do not complete differentiation into functional effectors, triggering a hyporesponsive state (3, 31). Exhausted T cells on the other hand are competent effector cells initially but then progressively lose function (3).

Typical characteristics of functionally exhausted T cells are their lack of ability to proliferate (3) and limited capacity to produce effector cytokines (24). T cell exhaustion starts with initial loss of ability to produce IL-2, perform cytolytic activity and proliferate. This is followed by an inability to produce other cytokines like IFN-γ (32, 33). This inability to perform effector functions can be reversed during earlier stages of exhaustion, however as the exhaustion progresses the dysfunction becomes more permanent (3).

1.3.1 Adenosine

The rapid growth of the tumour creates unfavourable conditions for the survival of normal cells. Under extreme hypoxic conditions, adenosine production in the extracellular matrix is promoted due to high levels of anaerobic glycolysis and the Warburg effect (where cancer cells metabolise using aerobic glycolysis rather than the more efficient oxidative phosphorylation pathway) (34). Under normal conditions adenosine, a purine nucleoside, is immunosuppressive and functions at relatively low concentrations comparable across different tissues (35). It plays an important part in homeostatic processes, such as vasodilation, mast cell activation and immunomodulation (36). In tumours, enzymes involved in deamination and adenosine phosphorylation are found in abundance. The presence of these enzymes therefore suggests that they may be potentially manipulated to control the adenosine concentration in the TME (37).

Anaerobic glycolysis in combination with the Warburg effect results in the abundance of AMP as well as increased CD73 expression by tumour cells (38). 5'nucleotidase, also known as CD73, is an ectoenzyme which catalyses the breakdown of AMP into adenosine (34, 39, 40). 5'nucleotidase further contributes to increased adenosine concentrations through the dephosphorylation of ATP into adenosine with the help of CD39 (another ectoenzyme) (41) as well as breakdown of extracellular adenine nucleotides (37, 40, 42, 43). Adenosine is

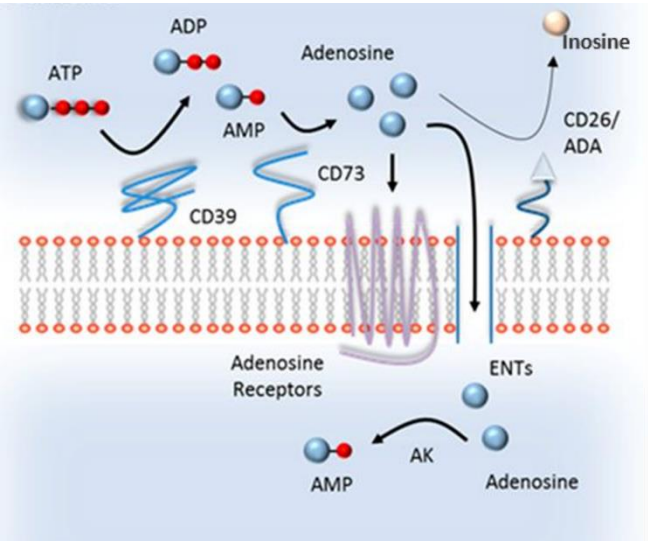

Figure 1.3 - The Adenosine Cycle

ATP is broken down into ADP by CD39 on the cell surface. ADP may also be further broken down by CD39 to AMP. AMP is then broken down to Adenosine by ectoenzyme 5'nucleotidase (CD73). Adenosine binds to adenosine receptors to activate them or is broken down by Adenosine Deaminase (ADA) into Inosine extracellularly. Alternatively, equilibrative nucleoside transporters (ENTs) can transport adenosine into the cell, where it is phosphorylated by adenosine kinase (AK) to AMP.

(Adapted from Bowser et al, 2018 (44))

metabolised by adenosine deaminase (ADA) and adenosine kinase, as displayed in Figure 1.3 (36, 40, 44).

Adenosine receptors comprise A1, A2A, A2B and the A3 receptors (45). These are metabotropic, G-protein-coupled receptors that work downstream via adenylyl cyclase (35, 46). They are further categorised into high-affinity (A1 and A2A), low affinity (A2B) and low abundance (A3) receptors (40, 47). Adenosine A2A receptor (A2AR) is most commonly found on immune cells and is considered a T cell surface CIR due to its inhibitory effect on T cells when bound by adenosine (34). It is considered a high affinity adenosine receptor due to its adenosine-binding at low concentrations (48). A2AR and A2B adenosine receptors (A2AB) are known to dampen immune effector functions as well as increase the prevalence and activity of immunosuppressive cell subsets when activated (41). Increased adenosine concentration leads to the upregulation of A2AR on the CTL surface, increasing their susceptibility to inhibition (34).

Concentrations of adenosine around tumours are between 10- and 20-fold higher than when compared to subcutaneous measurements in the same location (37). This elevation of adenosine in the TME impedes anti-tumour immunity (49). Adenosine influences cytotoxic T cell activation and effector functions by engaging the A2AR receptor on the T cell surface and thus interfering with their recognition of and adhesion to target tumour cells (37, 43). Adenosine has also been shown to inhibit the exocytosis of cytotoxic granules by natural killer cells as well as contributing to multi-drug resistance in glioblastoma treatment (25, 37). CD8+ T cells are more susceptible to the immunosuppressive effect of adenosine due to the relatively low ADA activity in CTLs which is found alongside CD26 on the T cell surface (50). CD26 is a T cell activation antigen which binds to ADA as it is released and mediates the effects of adenosine on the cell (51). Adenosine further inhibits anti-tumour CTL function in a variety of ways. When T cells undergo activation in the presence of adenosine, CD25 (a high-affinity IL-2 receptor) expression is lower than on cells that activated in the absence of adenosine. In addition, adenosine inhibits Fas ligand expression and decreases granzyme B expression of CTLs which interferes with their cytotoxic activity (43). Apart from this, adenosine has been shown to increase transcription and expression of 5’nucleotidase as well as inhibit adenosine kinase activity, creating an environment favouring adenosine synthesis (37, 43, 52). Under hypoxic conditions adenosine kinase has been shown to be inhibited which also further contributes to elevated adenosine concentrations in the TME (43).

1.3.2 Coinhibitory Receptors

Due to the wide range of immunosuppressive factors within the TME, CTLs are pushed towards an exhausted phenotype, impairing their anti-tumour activity and thus allowing the tumour to

continue to grow without clearance. Amongst their lack of ability to perform effector functions, exhausted CD8+ T cells are also characterised by the increased expression of CIRs on their surface (30, 32). These receptors are expressed transiently on the cell surface after CTL activation and when engaged with their ligands, CIRs inhibit CTL functions (53). It is important to note that whilst high CIR expression is associated with dysfunction, transient expression of CIRs is expected for activated cells to provide a means of controlling their activity. A lack of control of the CD8+ T cell cytotoxic mechanisms leads to excessive cytotoxic activity, the damaging consequences of which are demonstrated in autoimmune diseases such as multiple sclerosis (54). However, this regulatory mechanism has been utilized by tumours to enable them to evade immune clearance by the immunosuppressive factors in the TME leading to upregulation of CIRs on anti-tumour CTLs (2, 46).

Cytotoxic T-lymphocyte-associated protein 4 (CTLA4), is the first CIR to be identified on T cells. It is highly homologous to CD28 and bind B7 molecules with high affinity. When bound to its ligands, CTLA4 inhibits T cell proliferation and IL-2 production (53, 55).

PD1

Programmed cell death-1 (PD1) receptor is also expressed by CTLs. It is part of the CD28 family and is considered a hallmark for both T cell activation and exhaustion (56). PD1 is expressed on a variety of other cells, such as natural killer cells, B cells and activated monocytes, and is activated when bound by its ligands PDL1 and PDL2 (56-58). PDL2 expression is much more limited than that of PDL1. PDL1 is found on several immune cells, for example macrophages, DCs, B and T cells, as well as on tumour cells (58, 59). PDL1 is largely expressed on different solid tumours, such as breast, colon, liver, neck. High levels of PDL1 expression contribute to the immunosuppressive environment of tumours and acts in synergy with other factors to inhibit CTL function (58). For example, PDL1 is able to interact with B7.1, sending inhibitory responses to the T cells in a bidirectional manner due to the expression of both on immune cells like DCs, macrophages, B cells and T cells (60). Additionally, the IFN-γ production by T cells causes increased PDL1 expression on tumour cell surface, which upregulates and binds PD1 on the T cell surface creating an immunosuppressive loop (61). Once activated, PD1 protects T cells from overstimulation by acting via PI3 kinases and inhibiting the expression of transcription factors for effector functions, such as GATA3 and Tbet (15, 62, 63). It also significantly affects production of effector cytokines, e.g. IFN-γ and TNFα (56, 58). TILs have been shown to express higher levels of PD1 on their surface than T cells found in the peripheral blood and normal tissues, indicating the upregulation of this CIR by the tumour in the TME (24).

TIM3

PD1 has been shown to very often be co-expressed with T-cell immunoglobulin and mucin-domain molecule 3 (TIM3). When bound by its ligands, such as Galectin 9, TIM3 sends apoptotic signals to the cell, initiating its death (64). However, cell death appears to be triggered only at high levels of TIM3 engagement; when TIM3 is bound at low levels, this seems to convey inhibitory signals inciting CTLs into a dysfunctional state rather than cell death which explains how exhausted T cells persist in chronic conditions (64).

TIM3 upregulation is thought to hallmark extensively exhausted cells (32). Blockade of TIM3 has been shown to restore the proliferative and cytokine production ability of TIM3+ CD8+ T cells (64). Further, TIM3+ cells have been observed to display the highest levels of co-expression of other CIRs than the co-expression seen with other checkpoints. This highlights the role of TIM3 as a marker for CD8+ T cell exhaustion (65). In renal carcinoma and human melanoma, the expression of both PD1 and TIM3 together on CTLs has been correlated with a poor prognosis of said cancers (66, 67). PD1+TIM3+ CTLs found in tumours have been shown to display the most severe effector dysfunction than when compared to other combinations of CIR expression (64). TIM3 is also abundantly found on T_{reg} cells, where cells that expressed PD1 alongside TIM3 are highly immunosuppressive, further highlighting the inhibitory nature of this CIR combination (68).

LAG3

CIR lymphocyte activation-gene 3 (LAG3) inhibits T cell effector functions as well as cell expansion by interfering with downstream TCR signalling (33, 66, 69). It is commonly found on CD4+ and CD8+ T cells and due to its progressive expression on dysfunctional TILs has also been associated with CD8+ T cell exhaustion (64, 66, 70). High surface levels of LAG3 on the surface of CTLs have been correlated with a poor outcome in renal cell carcinomas (66). LAG3 localises next to the CD3-TCR complex on the T cell surface and is activated when bound by MHC II molecules on APCs (70). Once activated, it negatively regulates T cell activity and inhibits signal transduction via the TCR (71).

TIGIT

T cell immunoglobulin and immunoreceptor tyrosine-based inhibitory motif (TIGIT) has been strongly linked to immunosuppression. When bound by its ligands CD155 and CD112, it inhibits DC maturation and induces secretion of the immunoinhibitory IL-10 of immature DCs (72). CD155 binds TIGIT at high affinity, whilst CD112 does so with low affinity. Both molecules are involved in anti-tumour activity of T and natural killer cells (73) and are found on APCs as well as tumour cells, such as melanoma cells (72). TIGIT is expressed by T_{reg} cells, activated T cells and natural killer cells. Especially its immunoregulatory role in T_{reg} activity has been

highlighted by $TIGIT^{HIGH}$ T_{reg} cells being highly immunosuppressive. In addition to this, CTLs with high surface levels of TIGIT have been shown to be incapable of performing effector functions (74). Further, upregulation of TIGIT has been correlated with expression of PD1 on TILs (72).

Targeting CIRs in tumour therapies is an active area of research. Being able to interfere with the inhibition of anti-tumour CTLs would allow restoration of the immune system's defences and thus clearance of the malignant cells.

1.4 Immunotherapies

Immunotherapies provide a novel route to target malignancies in cancer treatment. The invasiveness and side effect profiles of traditional therapies are not applicable to treatments based on the host's own immune system. Cancer immunotherapies allow specific targeting of therapies, which further provide the potential of long-lasting immunity against cancer (75). A wide range of routes are investigated to restore and utilise endogenous anti-tumour activity. For example, using adoptive cellular therapy in which CD4+ T cells and CTLs are removed from the patient's blood or tumour, then expanded *ex vivo* to increase their numbers and returned to the patient in combination with different cytokines to fight the tumour. This is done in an attempt to counter the immunosuppression exerted by the tumour on immune cells and has been shown to elicit high response rates (49% to 72%) and longevity of tumour regression (76, 77). However, adoptive cellular therapy has several problems. For example, the depletion of T cells from the body leaves it susceptible to infections, especially when paired with radiotherapy as is often conventional in cancer treatment. The emergence of synthetic biology has allowed a different route to improving anti-tumour immunity by providing the ability to engineer CTLs. Central tolerance prevents autoimmunity of CD8+ T cells against healthy cells, however this mechanism also means that CTLs are often tolerised against tumour antigens. Furthermore, the few anti-tumour CTLs that do manage to escape central tolerance are not strongly activated against tumour-antigens and exhibit low-affinity TCRs. The transfer of TCR α and β chains specific for tumour-antigens aims to help effectively target CTLs to tumour tissue. This method allows for the engineering of the TCR to exhibit higher affinity for antigens (78).

Chimeric antigen receptors (CAR) aim to overcome central tolerance with a different approach. CARs are made by fusing the variable domain of immunoglobulin molecules to the constant domain of TCRs (including their intracellular signalling domains) and recognise target proteins on the cell surface without being dependent on display of tumour antigens via MHC molecules (79). Due to the much higher affinity of antibodies than TCRs for their targets, this provides anti-tumour CTLs possessing CARs with the potential to overcome central tolerance (76, 78). These

methods of T cell engineering have shown tumour regression in several cancers like melanoma and sarcoma (80).

A common aim of immunotherapies is to increase the number and strengthen the cytotoxic functions of endogenous CTLs present in the patient, since high quantities of TILs have been associated with good prognoses in several cancers, such as colorectal, renal, breast, oesophageal and non-small-cell lung cancer (81-85). This highlights the importance of therapies to establish strong and proliferative tumour-specific CTL responses. DC-based therapies target the professional APCs to improve their presentation of antigen to CD8+ T cells via MHC class I molecules and to ultimately initiate CTL proliferation. This may be done by loading patients' DCs with tumour antigens *ex vivo* and returning them back to the patient (86). *Ex vivo* culturing of DCs has shown clinical safety and lasting responses (87).

However, it is important to note that adoptive transfer of CTLs or their activation do not always suffice to eradicate the tumour, due to the tolerance to cancer antigens and the immunosuppression experienced by CTLs that occurs in the TME.

1.4.1 Targeting Immunosuppression

Another route to improve patients' own immune reaction against cancer cells is to target the immunosuppressive tactics employed by the tumour to prevent CTLs from performing their cytotoxic functions. As mentioned, high levels of extracellular adenosine can greatly contribute to the inhibitory nature of the TME. Therapies aimed at targeting adenosine are focussed on one of two routes, to either manipulate adenosine synthesis or adenosine signalling. This is mostly attempted using inhibitors in the form of small molecules but can also be done using blocking antibodies or pharmacological mediation (88, 89). Small molecules inhibiting A2AR and A2BR have been shown to inhibit and prevent the metastases of CD73+ tumours (89). A2AR antagonists are currently being investigated clinically in Parkinson's disease and exhibit great clinical safety suggesting that these agents could potentially be translated into cancer treatments (90).

5'nucleotidase also presents an attractive target for anti-tumour therapies due to its role in the production of adenosine, angiogenesis and role as a mediator for tumour and immune cell migration (41). Furthermore, 5'nucleotidase is expressed highly on cancer cells and cancer-associated fibroblasts alongside A2AR (34). Expression on tumour cells has been associated with increased tumour migration and invasive capability (88, 91). Thus, 5'nucleotidase carries great potential as a tumour biomarker to identify poor prognosis and metastases of tumours. However, although this aspect is highly researched, currently no fully human or humanised anti-5'nucleotidase antibodies have been generated for therapeutic use in the clinic (41, 92).

1.4.2 Targeting Coinhibitory Receptors

Studies have shown that monoclonal antibodies (mAbs), which block the signalling via CIRs, improve CTL functions *in vitro* and increase tumour killing *in vivo* in several animal models, displaying how CIR blockade can rapidly reinstate the effector functions of tumour-specific CTL (93-96). In clinical trials in which Iambrolizumab, a mAb that targets PD1, was given in patients with advanced melanoma, half of the cohort responded (102). Other trials using alternative anti-PD1 mAbs Pembrolizumab and Nivolumab were also very promising; these blockades displayed clinical efficacy and durability in a variety of cancer types (63). Nivolumab has provided long-term immunity in patients with melanoma and has also been shown to elicit strong responses in patients with renal-cell and non-small-cell lung cancer (97, 98). Indeed, in patients with PDL1+ non-small-cell lung cancer patients, treatment with Pembrolizumab gave rise to longer time periods without tumour progression, an overall better prognosis with fewer side effects than patients receiving chemotherapy (99). The development of these drugs provides a novel and highly effective alternative treatment of melanoma and non-small-cell lung carcinoma and both, Pembrolizumab and Nivolumab, are now FDA-approved therapies for these cancers (100, 101).

Despite this success, many patients receiving treatment with one type of CIR inhibitor do not always respond and many cancers have proven resilient to anti-CIR therapies (61, 63, 93, 95, 100, 102). It can prove challenging to target therapies to dysfunctional T cells via CIRs specifically, since these markers are also associated with T cell activation which means these markers are found also on functional T cells and exhibit versatile expression profiles. Due to its strong association with both activation and exhaustion, especially the development of TIM3-blockade has proven difficult (15). Furthermore, the complexity of CIR interaction also very likely contributes to the limited successes of CIR blockade therapies. The co-inhibitory system presents a variety of non-overlapping ways to achieve disruption of T cell functions and multiple inhibitory pathways are in existence which may cooperate to inhibit CTL effector functions. Their interactions are not well understood, thus blockade of one signalling pathway can cause the activation of different receptor signalling instead (32). The poor understanding of downstream mechanisms of CIRs further complicates the development of therapies against the receptors (32). Based on this non-redundancy of the CIR system, blocking of several CIRs at once is actively researched and has proven clinical efficacy. Blockade of TIGIT as well as PD1 increased CTL expansion and effector functions as well as CTL numbers in the periphery and the tumour site in advanced melanoma patients (72). Concurrent therapy of Ipilimumab (an anti-CTLA4 mAb) and Nivolumab elicited higher immune activity in melanoma patients than either therapy alone. The patients elicited rapid responses and moreover, the majority of patients displayed 80% or higher tumour regression after 12 weeks of treatment (63, 98, 103). The

combination of Ipilimumab and Nivolumab has also been shown to be effective in the treatment of non-small-cell lung carcinoma; patients with PDL1+ tumours displayed increased and durable response rates (104). However, the potential toxicity of combining the blockade of several CIRs has been questioned. As more agents are being used, more cases of immune-related adverse events (IRAE) have been reported than in therapies using one CIR blocker only. These included thyroid dysfunction, pneumonitis and dermatologic effects (63, 105, 106). In the study assessing the combination of Ipilimumab and Nivolumab in melanoma patients conducted by Wolchok *et al* (2013) ~50% of patients experienced IRAE from the treatment. Nonetheless, these responses were similar to those experienced in monotherapy alone and were generally reversible (98). Despite the success of multiple CIR blockade in some cancers, many patients still do not respond these therapies. Furthermore, several cancers have been shown to be resistant (107, 108).

The recent advances and successes in CIR blockade highlight the significance of their potential as cancer immunotherapeutic targets. Nevertheless, the variability in responses seen in patients also underlines the importance of investigating the expression patterns of receptors to be able to target them therapeutically (3). This knowledge is also crucial in terms of identification of biomarkers to predict the prognoses of different tumours (15).

In an aim to try to increase the efficacy of immunotherapies, several approaches to improve tumour immunity are also being tested in tandem. For example, it was found that the combination of A2AR blockers as well as inhibition of CIRs showed great success (34). This co-blockade increased CTL effector function and reduced metastases of melanoma and mammary tumours in mice (41). Further, the blockade of A2AR as well as low CD73 expression reduced expression of PD1 significantly, highlighting that targeting adenosine prior to anti-PD1 treatment may increase the efficacy of treatment by increasing the ratio of antibody:target (41). Based on the recent success of multiple CIR blockade, this route is being explored in combination with traditional cancer treatments like chemotherapy. The combination of Nivolumab and Ipilimumab with chemotherapy in the treatment of non-small-cell lung carcinoma is currently being explored in several Phase III clinical trials (104). These promising findings from initial trials of different agents highlight the importance of combining different strategies to restore efficient anti-tumour immunity. However, it is important to take dose-titrations of treatments into account to prevent IRAEs from summated immune toxicities when combining several therapies (109).

1.4.3 Tumour Vaccines

Tumour vaccinations provide a different route to improving the host's anti-tumour responses. Vaccination with tumour antigens or agents to better the presentation of tumour antigens in order to boost the anti-tumour response are amongst the most researched tumour vaccine methods (15). The targets of anti-cancer vaccines are highly variable. However, as mentioned, a highly desirable outcome is the proliferation of strongly primed anti-tumour effector CD8+ T cells to fight the tumour and develop memory T cells. Thus, DCs are commonly targeted to improve the presentation of tumour-antigen. As professional APCs these are the most efficient at driving strong proliferative responses of CTLs. Additionally, the delivery of the vaccination may be targeted into the cross-presentation pathway of DCs to elicit CD8+ T cell responses. Provenge, an FDA-approved vaccine, is used in the treatment of prostate cancer. By delivering the prostate cancer antigen prostate acid phosphatase (PAP) along with granulocyte-macrophage colony-stimulating factor (GM-CSF; a stimulant of DCs which is essential to ensure their maturation), it initiates DC activation and antigen presentation, leading to the activation of PAP-specific CTLs. Development of Provenge has allowed increased survival of patients with prostate cancer (110). A different approach using DCs is the genetic engineering of cancer cells to express GM-CSF in GVAX vaccines. Production of GM-CSF recruits and activates APCs, including DCs, and encourages internalisation of the tumour cells for cross-presentation to CD8+ T cells (111). These therapies have shown clinical efficacy in a variety of cancers (112, 113).

A limitation of tumour vaccines is the difficulty in identifying tumour-specific antigens which are not found on healthy tissues in order to prevent side effects (114). Melanoma is one of the few cancers against which effective vaccinations have been developed, due to malignant lesions consistently expressing certain antigens and thus enabling efficient CTL effector function in response to vaccination (109). The potential need for adjuvants, ensuring correct delivery to the target and tolerization to the delivered agent however are amongst the many other obstacles in the way of developing the ideal cancer vaccine (75).

1.5 SAGEs

Self-assembling peptide cages (SAGE) are synthetic, flexible networks formed by the assembly of short *de novo* coiled-coil peptides. These are formed by two complementary hubs, hub A and hub B. The trimeric hubs are created upon mixing of a homotrimer with either heterodimer A or B and the formation of disulphide bonds between them. Hub A and hub B are then mixed to build SAGEs, as shown in Figure 1.4. Further sequences can be added to SAGEs via their C-

terminus. Full proteins may be encapsulated by these networks, enabling the addition of localising proteins (e.g. GFP) to SAGEs (115). The possibility of exchanging the components of SAGEs allows alteration of their physical properties, such as size and charge, and makes them highly customisable to encapsulate a variety of material, such as enzymes or peptides. SAGEs provide an alternative method of delivering tumour vaccines and carry great potential to initiate desired antigen-specific responses.

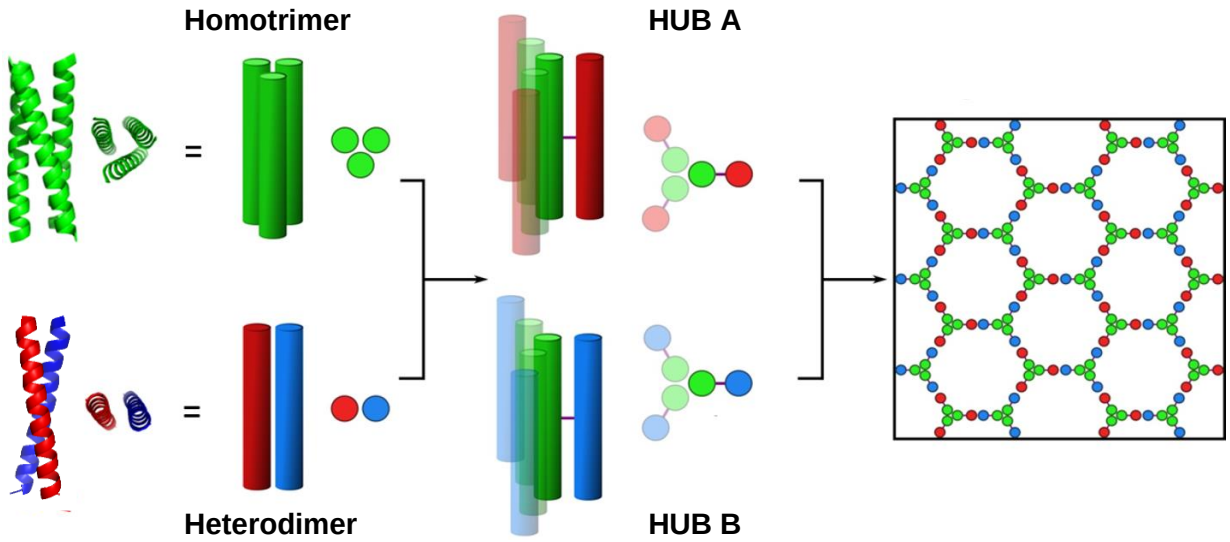

Figure 1.4 - The Assembly of SAGEs

SAGE molecules are built by the assembly of the hub A and hub B building blocks. These blocks in turn are made of two separate *de novo* peptides, a homotrimer (CC-Tri3) and a heterodimer (either CC-Di-A or CC-Di-B). Upon mixing of the homotrimer with one of the heterodimers, they bind through disulphide bonds and create Hub A and Hub B. Subsequent combination of the two hubs leads to their co-assembly into hexagonal networks which fold to form SAGE nanoparticles. *Adapted from Fletcher et al, 2013* (117)

Vaccine delivery methods such as subunit vaccines (in which highly immunogenic portions of pathogens are used), are usually limited in terms of components that can be administered via e.g. liposomes. In the meantime, a recent study by Morris *et al* (*unpublished; (116))* demonstrates that SAGEs are highly variable, non-toxic and immunogenic, and their modular nature allows interchangeability of components as well as the potential of protein attachment to the surface for precise targeting of a vaccine (116, 117). SAGEs also allow for the inclusion of several antigens together to deliver multivalent vaccines (75, 116). It has also recently been demonstrated that SAGEs are internalised by APCs *in vitro* which further highlights their potential as a DC-targeting agent to prime CD8+ T cells and elicit proliferative responses. Furthermore, the ability of SAGEs to trigger CD4+ T cell and B cell responses to specific antigens *in vivo* has been shown, demonstrating their capabilities in an immunological setting (116).

Because of these characteristics, especially the ability to add molecules to their exterior, SAGEs could be used in a tumour vaccination setting where encapsulated material can be targeted to DCs and into the cross-presentation pathway, to drive strong CTL proliferation and thus increase the number of tumour-specific, strongly activated CTLs.

1.6 CL4 Model

To study the immunosuppressive characteristics of tumours as well as the immune response against them, our lab uses BALB/c clone 4 TCR transgenic mice. This model was created by infecting B10.D2 mice with the influenza virus A/PR/8 H1N1 (PR8) and injecting isolated TCR cDNA from CTLs of the infected mice into fertilised mouse oocytes. Matured mice were then backcrossed for 5 generations to strengthen the genetic background (C57BL/6 X BALB/c) (118). The clone 4 (CL4) model thus expresses transgenic CL4 CD8+ T cells with TcR, composed of variants Vα10 and Vβ8.1, which are specific to the immunodominant K^d-restricted HA epitope (^{518}IYSTVASSL^{526}) and bind it with high affinity (119).

Renal carcinoma (Renca) wildtype cells were generated from BALB/c mice (120). These murine cells were transfected using pIRES-HA plasmid which encodes the haemagglutinin (HA) gene from PR8 to create the Renca HA cell line which is recognised by TcR transgenic CL4 CD8+ T cells (120). Subcutaneous injection of RencaHA cells into BALB/c mice gives rise to solid carcinomas which express the HA protein and K^dHA peptide complexes which can be used to study the properties of the TME. Adoptive transfer of naïve CL4 CD8+ T cells into RencaHA tumour-bearing BALB/c mice results in their activation through cross-presentation of K^dHA epitopes by DCs within tumour-draining lymph nodes. Low avidity CD8+ T cells become tolerised and only a very small amount infiltrates the tumour, whilst more TILs were developed when high avidity CL4 CD8+ T cells were primed in the tumour-draining lymph node (TDLN;(121)). However, once CL4 effector CTL travel to the site of the tumour, no tumour-killing is undertaken and the tumour continues to grow (121). Further examination of the CL4 TIL revealed a loss of CTL function, including the inability to produce IFN-γ (121).

Later studies in our lab found that in the TME expression of 5'nucleotidase and CD39 is increased on a variety of infiltrating cells which has the capacity to contribute to the elevated adenosine levels in the TME. Most notably, high levels of 5'nucelotidase and CD39 on the surface of CD4+CD25+FoxP3+ T_{reg} cells were significant, since these cells were highly abundant in the TME but not in TDLN (illustrated in Figure 6.1 in the Appendix). Additionally, it was found that co-culturing of these T_{reg} cells with CL4 CD8+ T cells *ex vivo* lead to complete suppression of CL4 CD8+ T cell effector function, indicating that the tumour-infiltrating T_{reg} cells could directly inhibit CL4 CTL function. However, the addition of an adenosine receptor

antagonist into this culture found significant restoration of CL4 CD8+ T cell proliferation. These findings indicate that T_{reg} cells are able to mediate suppression of CL4 CD8+ through the action of adenosine.

1.7 Hypotheses

Several factors in the TME affect anti-tumour CD8+ T cells and suppress their functions to promote an exhausted phenotype. We hypothesise that the upregulation of co-inhibitory receptors on the surface of CD8+ T cells is related to the increased concentration of adenosine experienced by the cells in the TME. Further, we hypothesise that the use of SAGE particles can improve the CD8+ T cell proliferative response by allowing cross-presentation of delivered peptide with MHC class I molecules.

Hypothesis 1:

Adenosine dampens the proliferative ability of CD8+ T cells *in vitro*

Experimental Approach:

i. Culture CD8+ T cells with varying concentrations of NECA *in vitro* and examine how this affected their proliferation compared to untreated cells.

Hypothesis 2:

Adenosine increases the expression of coinhibitory receptors
on the CD8+ T cell surface *in vitro*

Experimental Approach:

i. Assess how expression of TIM3, TIGIT, LAG3 and PD1 changes *in vitro* as CD8+ T cells activate using a pulsed splenocyte reaction or anti-CD3 and anti-CD28 monoclonal antibodies
ii. Determine the changes in these receptors when CD8+ T cells are cultured *in vitro* in the presence of different concentrations of NECA.

Hypothesis 3:

Conjugated SAGE particles can trigger CD8+ T cell activation and proliferation *in vivo*

Experimental Approach:

i. Test the ability of various HA peptides to prime CD8+ T cells alone *in vitro*
ii. Assess the ability of conjugated peptide-SAGEs to prime CD8+ T cells *in vivo*
iii. Determine whether conjugation of HA peptides to SAGE improves the CD8+ T cell proliferative response *in vivo* compared to peptides alone

Chapter 2 - Materials and Methods

2.1 MATERIALS

2.1.1 Buffers

2.1.1.1 MACS Buffer

MACS Buffer was used for magnetic-activated cell sorting. It was prepared by adding 0.5% (w/v) Bovine Serum Albumin (BSA; Sigma-Aldrich, St. Louis, USA) and 2mM EDTA (Sigma-Aldrich) to 1x Phosphate buffered saline (PBS; Thermo Fisher Scientific, Waltham, USA). The solution was then filtered through a 0.45 µm filter (Sartorius Stedim Biotech, Goettingen, Germany) and stored at 4˚C.

2.1.1.2 FACS Buffer

FACS Buffer was used for cell surface staining. FACS Buffer consisted of 0.5% (w/v) BSA in PBS which was then filtered through a 0.45 µm filter (Sartorius Stedim Biotech) and stored at 4˚C.

2.1.2 Cell Culture Reagents

2.1.2.1 Complete Medium (CM)

CM contained RPMI 1640 with L-Glutamine (Thermo Fisher) supplemented with 10% (v/v) fetal bovine serum (FBS; Lot 1851515; Thermo Fisher), 50 U/ml penicillin/streptomycin (Thermo Fisher) and 5 $x10^{-5}$ M b-Mercaptoethanol (Sigma-Aldrich).

2.1.2.2 IL-2 Medium

IL-2 medium was prepared in a similar manner to CM, with the addition of 0.1% (v/v) hIL-2 (NCI Preclinical Repository, Maryland, USA).

2.1.3 Mice

6 to 8-week old $Thy1.1^{+/+}$ $CL4^{+/-}$ BALB/c TCR transgenic (CL4) mice (specific to dominant K^{d}HA peptide (^{518}IYSTVASSL^{526}); (118)) and $Thy1.2^{+/+}$ BALB/c mice were used. Mice were bred in specific pathogen-free conditions at the University of Bristol Animal Services Unit. All experiments were conducted in accord with current UK Home Office guidelines. Mice were culled by either cervical dislocation or exposure to gradually elevating CO_2.

2.1.4 P815 Mastocytoma Cells

Non-adherent P815 Mastocytoma cells were purchased from ATCC (Manassas, USA).

2.1.5 Passaging of Renca and P815 Cells

Adherent Renca and non-adherent P815 cells were maintained in CM.

Adherent Renca cells were routinely passaged at 70 - 80% confluency. Tissue culture CM was removed and cells were washed with PBS. Following this, cells were incubated with 2 ml of 1X Trypsin – EDTA solution (Sigma-Aldrich) at 37˚C for 3 minutes to detach the cells. Trypsin was then neutralised with 5 ml of CM and cells were collected into 15 ml falcon tubes (Corning, New York, USA) and centrifuged at 1200 rpm for 3 minutes. Pelleted cells were then resuspended in CM, seeded into either new T25 or T75 cm^2 tissue culture flasks at suitable split ratios and kept in a humidified incubator at 37˚C and 5% CO_2 atmosphere.

Passaging of non-adherent P815 cells was performed at 70 - 80% density by removing a portion of cells from the culture flask. The removed volume was replaced with CM for an appropriate seeding density and tissue culture flasks were kept in a humidified incubator at 37˚C and 5% CO_2 atmosphere.

2.1.6 A/PR/8/H1N1 Virus

A/PR/8/H1N1 influenza virus was prepared in house.

2.1.7 Antibodies for Flow Cytometry

A range of monoclonal antibodies (mAbs) specific to murine extracellular and intracellular molecules were applied. All antibodies used for staining for analysis by flow cytometry were conjugated directly to fluorochromes. All antibodies were titrated and optimal concentrations (highest dilution of antibody still giving a clear separation between positive and negative populations) were chosen. In most cases, a stain index was also calculated to compare the difference between positive and negative signals. Further information of the antibodies used, including their staining concentrations used, can be found in the appendix (Table 6.1). In staining panels using several antibodies, and thus several fluorochromes, Fluorescence Minus One (FMO) controls were prepared. These contain all antibodies used except for one fluorochrome-conjugate which allows representative gating and interpretation of flow cytometric data.

2.1.8 Peptides

Peptides synthesis was performed at the University of Bristol BrisSynBio facility using a CEM Liberty Blue peptide synthesiser. Peptides were purified using high performance liquid chromatography (HPLC) and identified by MALDI-TOF mass spectrometry. Peptide purity (>85%) was analysed by RP–HPLC. Peptides were then freeze-dried and stored at -20˚C in

powder form. Just prior to use, peptides were reconstituted in PBS and concentration was adjusted accordingly.

The following peptides were used and were kindly provided by Caroline Morris (School of Chemistry, University of Bristol):

EPITOPE	H-IYSTVASSL-OH
POL	H-AVGAGATAEEIYSTVASSL-OH
LEADER	H-EKLAGFGAVGAGATAEEIYSTVASSL-OH

Constituents of SAGE particle:

CC-Tri3	Ac-G EIAAIKK EIAAIKC EIAAIKQ GYG-NH
CC-Di-A	Ac-G EIAALEK ENAALEC EIAALEQ GWW-NH
CC-Di-B	Ac-G KIAALKK KNAALKC KIAALKQ GYW-NH

The self-assembling peptide cage (SAGE) particle consists of a mixture of a homotrimer (CC-Tri3) and a heterodimer (CC-Di-A + CC-Di-B). Combination of the particles forms complementary hub A (CC-Tri3-CC-Di-A) and hub B (CC-Tri3-CC-Di-B) constructs which serve as SAGE building blocks. When mixed, association of CC-Di-B and CC-Di-A in the constructs leads to co-assembly of the hubs and thus the formation of the SAGE particle (117). Functionality is introduced into the SAGE by adding to the C-terminus of the homotrimer, in this case using the above POL sequence.

POL-SAGE TRIMER (functionalised via homotrimer):
Ac-GEIAAIKKEIAAIKCEIAAIKQGYGGGAVGAGATAEEIYSTVASSL-OH

2.2 METHODS

2.2.1 Mice

Treatments described below that involved direct handling of mice were kindly performed by Dr Grace Edmunds and Dr David Morgan.

2.2.1.1 Genotyping of Mice

Blood samples from tail veins of weaned CL4 mice were stored in PBS supplemented with heparin to avoid clotting. Peripheral blood was stained with fluorescently-labelled mAbs against CD8α and Vβ8.1 to type for CL4 TCR gene expression (shown in Figure 6.2 in the Appendix). Erythrocytes were lysed by adding 1 ml of Ack Lysing buffer (Thermo Fisher) for 3 minutes at RT. Lysis was stopped by adding 10 ml CM.

2.2.1.2 Adoptive Transfer of CL4 CD8+ Cells

In some instances, BALB/c mice were inoculated intravenously with 3-5 x10^6 naïve, MACS-purified and CTV-labelled (described *below*) CL4 CD8+ T cells in 100 µl PBS.

Mice were left for 4 or 8 days, then spleens, and in some cases lymph nodes, were collected for further investigation.

2.2.1.3 Infection with Influenza for in vivo Experiments

Treatment of mice was assigned using precautions to eliminate batch effects by numbering mice and randomly assigning treatments to numbers.

Following Adoptive Transfer, mice received intraperitoneal injections to administer approximately 1200 HA units of influenza virus strain A/PR/8/H1N1.

Mice were left for 3 or 7 days, then spleens were collected for further investigation.

2.2.1.4 Inoculation with SAGE particles for in vivo Experiments

Treatment of mice was assigned using precautions to eliminate batch effects by numbering mice and randomly assigning treatments to numbers.

Following Adoptive Transfer, Self-assembling peptide cage (SAGE) particles were injected subcutaneously with 1 mM of peptide material in 100 µl PBS into the dorsal neck-scruff of mice.

Peptide-only treatments were administered in an identical manner.

Mice were left for 4 or 8 days, then spleens were collected for further investigation.

2.2.2 Counting Cells using Haemocytometer

Cells were counted and assessed for viability using a haemocytometer counting chamber (Hawksley, Sussex, UK). An aliquot of the cell suspension was mixed with an equal volume of trypan blue solution (GE Healthcare, Chicago, USA) and transferred into the counting chamber. Dead cells were identified by their blue colour, whilst live cells remained bright. Live cells were counted and total cell number was calculated using the following equation:

$$\text{Total cell \#} = \text{Average Cell Count} \times \text{Dilution Factor} \times 10^4$$

2.2.3 Storage and Retrieval of Cells from Liquid Nitrogen

To freeze, cells were resuspended in cold freezing medium containing 10% (v/v) dimethyl sulphoxide (DMSO; Santa Cruz Biotechnology, Dallas, USA) in FCS. Freezing was performed at maximum 5 x10^6 cell per 1 ml in Cryo.S™ cryovials (Greiner Bio-One, Kremsmuenster, Austria) which were then placed in a Nalgene Freezing Container filled with isopropanol (Fisher Scientific, Loughborough, UK) overnight. The next day, cryovials were moved to liquid nitrogen for long-term storage.

To retrieve cells from liquid nitrogen, they were quickly thawed at 37˚C and diluted in 20 ml CM. After centrifuging at 1200 rpm for 5 minutes the pellets were resuspended gently using CM. Following this, the cells were transferred into T25 tissue culture flasks and kept in a humidified incubator at 37˚C and 5% CO_2 atmosphere.

2.2.4 Tumour Cells

2.2.4.1 Irradiation of Renca Cells

Renca cells were irradiated prior to peptide pulsing. Renca cells were harvested and counted. Cells were resuspended at 1 x10^6 cells/ml in CM and irradiated with 9600 Rads for 2.5 hours using a Cs^{137} source of gamma irradiation unit (RX30/55; Gravatom Projects, Grosport, UK). Following this, the cells were washed in RPMI 1640, centrifuged and re-suspended in CM.

2.2.4.2 Mitomycin C Treatment of P815 cells

P815 cells were treated with Mytomycin C prior to peptide pulsing. Mitomycin C (Sigma-Aldrich) was added at a final concentration of 2 µg/ml to 70 - 80% dense cultures and incubated at 37˚C and 5% CO_2 atmosphere overnight. The following day, cells were washed in RPMI 1640, centrifuged and re-suspended to 1 x10^6 cells/ml in CM.

2.2.4.3 Peptide Pulsing of Tumour Cells

Renca and P815 cells were prepared for peptide pulsing as described in section 2.2.4.1 and 2.2.4.2, respectively. The chosen peptide concentration was added into prepared cells and incubated for 1 hour in a humidified incubator at 37˚C and 5% CO_2 atmosphere. The pulsed cells were then washed twice and resuspended in CM for culturing with naïve CD8+ T cells.

2.2.5 CL4 CD8+ T Cells

2.2.5.1 Isolation of Lymphocytes from Peripheral Lymphoid Tissue

Hon Lam kindly assisted often in the processing of spleens and subsequent experimental *in vitro* treatments.

Spleens were collected from CL4 mice using sterile instruments and kept in RPMI. Spleens were cut into small pieces with sterile scissors and, using sterile syringe plungers, were mashed through 40 µm cell strainers (Corning Falcon, Corning, USA) into 35 mm Cell Culture Dishes (Corning). This was repeated several times while washing with CM. The cell suspension was collected into a 50 ml conical tube and the Petri dish was rinsed with CM. Red blood cells were lysed using 1 ml Ack Lysing Buffer (Thermo Fisher) per spleen used. Lysis was stopped after 3 minutes by adding 10 ml CM and 10 ml PBS.

2.2.5.2 Magnetic-Activated Cell Sorting

CD8+ cells were positively enriched using Magnetic-activated cell sorting (MACS). The lymphocyte mixture was magnetically labelled using anti-CD8a MicroBeads (Miltenyi) by resuspending cells in 45 µl MACS buffer and 5 µl microbeads per 10 x10^6 cells and incubating for 30 minutes at 4˚C. CD8a+ cells were then isolated using the Miltenyi MidiMACS Separator, MidiMACS LS columns (Miltenyi) and MACS buffer. Unbound beads were removed by adding 10 ml cold MACS buffer and centrifuging for 5 minutes at 1400 rpm at 4˚C. Cells were resuspended in cold MACS buffer, applied to a LS separation column that was previously washed with 6 ml MACS buffer and placed into a MidiMACS Separator. Re-application of run-through eluent was performed to minimize loss of CD8+ cells. The column was washed with 9 ml MACS buffer and removed from the magnet. Magnetically-labelled cells in the column were flushed out by forcing 6 ml MACS buffer through with a plunger. Enrichment Purity of this method was routinely tested by taking a sample before magnetic labelling and one after MACS, followed by staining with Vβ8.1 and CD8α mAbs (see *Cell Surface Staining* below). Purity was routinely ~95% (shown in Figure 6.3 in the Appendix).

2.2.5.3 Labelling with CellTrace™ Violet

In proliferative assays, cells were labelled with the cell tracking dye CellTrace™ Violet (CTV; ThermoFisher). Vials were reconstituted with 20 µl DMSO and stored at -20˚C. Purified CL4 CD8+ T cells were pelleted and resuspended at 4 x10^6 cells/ml in PBS, then mixed with 1 µl/ml CTV and incubated in the dark at 37˚C for 20 minutes. Cells were quenched with CM and were let to settle for 15 minutes at room temperature and spun down. For *in vitro* cultures cells were resuspended at 1x10^6 cells/ml in CM, while for *in vivo* experiments cells were resuspended at 3-5x10^6 cells/ml in PBS.

2.2.6 Priming in vitro

2.2.6.1 CL4 CD8+ T cell Priming with Anti-CD3 plus Anti-CD28 Monoclonal Antibodies

CL4 naïve T cells obtained from MACS were activated using plate-bound anti-CD3 *in vivo* mAb (145-2C11; BioXCell, Lebanon, USA) and soluble anti-CD28 *in vivo* mAb (37.51; BioXCell). 24-well plates (Corning) were coated by adding 250 µl of 10ug/ml anti-CD3 mAb per well and leaving overnight at 4˚C. The following day, unbound antibody was removed and 1x10^6 purified, in some cases CTV-labelled CL4 T cells were added to each well. Additionally, 250 µl soluble anti-CD28 was added to each well for a final assay concentration of 1ug/ml. Cells were cultured for either 48, 72 or 96 hours in CM, and maintained in a humidified incubator at 37˚C and 5% CO_2 atmosphere.

2.2.6.2 CL4 CD8+ T cell Priming using Peptide-Pulsed Tumour cells

Naïve CL4 T cells obtained from MACS and labelled with CTV (described above) were co-cultured directly with peptide-pulsed Renca or P815 cells. T cells were mixed with tumour cells 1:10 in flat-bottomed 24-well plates. Cells were cultured for 48 or 72 hours in CM and maintained in a humidified incubator at 37˚C and 5% CO_2 atmosphere.

2.2.6.3 Pulsed Splenocyte reaction (PSR)

Spleens were collected and macerated as before. Following erythrocyte lysis, cells were stained with CTV (*above*). 4 -5 x10^6 cells were added into each well of a 24-well plate in CM. Cells were then pulsed with the chosen concentration of peptide overnight. The next day cells were collected, washed 5 times with RPMI 1640 to remove free peptide and resuspended in IL-2 media at 4-5 x10^6 cells per well. On subsequent days fresh IL-2 media was added where necessary. Cells were cultured for 48, 72 or 96 hours in a humidified incubator at 37˚C and 5% CO_2 atmosphere.

2.2.6.4 NECA Treatment of CL4 cultures

In some experiments, CL4 T cell cultures were treated with pan-adenosine receptor agonist 5′-(N-Ethylcarboxamido) adenosine (NECA) (Sigma-Aldrich). NECA in DMSO was added to the culture at either 1 µM, 5 µM, 10 µM, 20 µM or 50 µM final assay concentration. NECA was added either at day 0 when CL4 T cells were naïve or after 24 or 48 hours of culture.

2.2.7 Fluorescence-Activated Cell Sorting (FACS)

Cells were stained for viability and cell surface markers in either round-bottom FACS tubes (Corning) or 96-well V-bottom plates (Thermo Fisher).

2.2.7.1 Viability Staining

Zombie NIR™ Fixable Viability Kit (Biolegend) was used to distinguish dead cells from live cells. The kit was brought to room temperature, 100 µl DMSO were added to one vial of dye for reconstitution and the vial was stored at -20˚C. Viability staining was carried out at a 1:100 concentration of 1 µl prepared dye per 1x10^6 cells. The dye was mixed with the cells and after 15 minutes incubation at room temperature in the dark, cells were washed twice using FACS buffer.

2.2.7.2 Cell Surface Staining

Cell surface staining was performed using fluorescently labelled mAbs. Where needed, non-specific binding of mAbs to Fc receptors was blocked using mouse BD Fc Block™ (2.4G2; BD Biosciences) by mixing with the cells, incubating for 15 minutes at 4˚C and washing with FACS buffer. Cells were then spun down for 5 minutes at 1400 rpm and resuspended in fluorochrome-

conjugated mAbs using the optimum concentrations as listed in Table 6.1. After 30 minutes incubation in the dark at 4˚C, cells were washed twice using FACS buffer and fixed in 1% PFA.

2.2.7.3 Fluorescence-Activated Cell Sorting (FACS)

Stained samples were fixed with 1% paraformaldehyde (PFA) until analysis. Fluorochrome-labelled cells were acquired using LSRII, Fortessa X 20 with Diva software (BD Biosciences) or Novocyte with NovoExpress software (ACEA Biosciences). Data was analysed using FlowJo™ software v10.3 and FMOs were used for gating (FlowJo, Ashland, USA).

2.2.8 Statistical Analysis

Statistical analysis was carried out using IBM SPSS Statistics v25 (IBM, New York, USA). Values of $p \leq 0.05$ were deemed significant, while $p > 0.05$ was considered not significant. To compare two groups, unpaired t-test was used, while for three or more groups one-way or two-way ANOVA was used as indicated. For multiple comparisons between groups, further analyses were conducted. Microsoft Excel was used in the analysis of some experiments. All graphs were created using GraphPad Prism software v7.04 (GraphPad, California, USA). Error bars on graphs indicate ±SEM.

Chapter 3 - The Effect of NECA on CD8+ T Cells

3.1 Background

Once CD8+ T cells have been primed against tumour-specific antigens and activated, they migrate to the site of the tumour to infiltrate it and attempt to eliminate the malignant cells. However once there, they enter a tumour-tolerant state and whilst activated, do not kill any cancer cells. This is due to the TME and its extreme immunosuppressive conditions. High levels of extracellular adenosine, amongst other immunosuppressive factors, largely contribute to the non-functional and exhausted phenotype of CD8+ T cells which display an inability to proliferate or produce effector cytokines IL-2 and IFN-γ.

Adenosine is of immunosuppressive nature; by recruitment of immunoregulatory cells (e.g. T_{reg} cells) and upregulation of anti-inflammatory factors, at moderate concentrations it plays an important role in the modulation of immune responses (36, 43, 122). Many immune cells express the adenosine receptor A2AR, which when activated by adenosine acts as a negative regulator of their functions (49). High rates of ATP and AMP hydrolysis in the TME lead to accumulation of high adenosine concentrations which interfere with the anti-tumour ability of CTLs.

Whilst under normal conditions CIRs are expressed to prevent potentially harmful overactivity of T cells, T cell exhaustion is associated with increased expression of receptors like PD1, TIM3, TIGIT and LAG3 on the CD8+ T cell surface (67). Upregulation of these receptors has been shown to correlate with poor prognoses of several cancers, such as renal cell carcinomas (123). Different combinations of CIR expression also play an important role in the success of cancer treatments (94, 124). Co-expression of TIM3 and PD1 has been observed on TILs in the TME from patients with cancers like metastatic melanoma, non-small cell lung and renal cell carcinoma (68). This combination is most commonly found on exhausted TILs. PD1+LAG3+ CD8+ T have also been shown to be more dysfunctional than exhausted PD1+ or LAG3+ only CD8+ T cells (125). Aside from this, CIR co-expression plays an important role in the immunosuppressive nature of the TME. TIM3+PD1+ T_{reg} cells have been found to be highly immunosuppressive, highlighting the suppressive power of this receptor combination.

Due to the rapid metabolism of adenosine by adenosine deaminase in an experimental setting (126), in these investigations 5′-(N-Ethylcarboxamido) adenosine (NECA) was used. NECA is a synthetic adenosine analogue which cannot be hydrolysed and acts as an A1 and A2 receptor agonist, with equal affinity for both receptors.

3.2 Aims

I. To assess the effect of NECA at different concentrations on the expression of TIM3, TIGIT, LAG3 and PD1 on CD8+ cells
II. To examine the effect of NECA on the proliferation of CD8+ T cells and if this varies depending on the addition time of NECA to the culture
III. To investigate the changes in expression of TIM3, TIGIT, LAG3 and PD1 on CD8+ T cells as they activate *in vitro* using either anti-CD3 and anti-CD28 mAbs or a pulsed splenocyte reaction

3.3 How does NECA influence CD8+ T cell proliferation?

Exhaustion of anti-tumour CD8+ T cells is characterised by their inability to perform effector functions, such as secretion of effector cytokines IL-2 and IFN-γ, as well as loss of their ability to proliferate. High extracellular levels of adenosine inhibit effector function of CTLs by interfering with their recognition of and adhesion to target cells (37, 43). Additionally, engagement of the A2AR on CTLs has been shown to inhibit their ability to perform cytolytic functions (25). Thus, since adenosine is a key contributor to the immunosuppressive nature of the TME, it was interesting to assess how different concentrations of NECA would influence the ability of CD8+ T cells to proliferate.

3.3.1 Investigating the effect of NECA on the proliferation of CD8+ T cells

To determine whether or not NECA affects the proliferation of CD8+ T cells, naïve CD8+ T cells were purified using MACS and CTV labelled. Cells were then primed with anti-CD3 and anti-CD28 mAbs and cultured in the presence of 1 µM, 2 µM, 5 µM, 10 µM, 20 µM or 50 µM NECA for 72 hours. Harvest and staining using specific fluorochrome-labelled mAbs was performed in 24-hour intervals (Figure 3.1). Proliferation past the first division was then analysed as shown in Figure 6.4.

As shown in Figure 3.1, no statistically significant difference in proliferation was found between different NECA concentrations at different time points using two-way ANOVA ($p \geq 0.05$). There was no proliferation between 0 and 24 hours (A) as cells were still naïve. At 48 hours (C) as cells activated and proliferation increased rapidly, analyses showed that there was no significant difference between NECA-treated and untreated wells. Between 48-hour and 72-hour time points an increase to ~85 % was observed in NECA-treated cells, so that at 72 hours (D) cells treated with either 1 µM, 5 µM, 10 µM, 20 µM or 50 µM of NECA had proliferated without any statistically significant differences to untreated control cells. Thus, the statistical analysis of the data indicated that NECA does not affect CD8+ T cell proliferation at any of the 5 concentrations. However, it is important to note that despite the lack of statistical confidence, graph (C) suggests

that the proliferation of CD8+ cells was impaired most (10 % proliferated cells) at 48 hours when treated with 50 µM NECA. ~30 % of CD8+ T cells had proliferated when cultured in the presence of 1 µM, 5 µM, 10 µM or 20 µM NECA, compared to nearly 60 % in untreated cells. As shown in (D), by 72 hours untreated and NECA-treated CD8+ T cells had highly proliferated; ~ 90% of untreated CD8+ T cells had proliferated, and 85-90 % of cells treated with 1 µM, 5 µM, 10 µM or 20 µM NECA had proliferated. Also ~75 % of CD8+ T cells cultured in the presence of 50 µM NECA had proliferated. This suggests that the presence of NECA during activation of CD8+ T cells elicits a lag in their proliferation, however by 72 hours cells had recovered and displayed similar levels of proliferation as untreated cells. CTLs undergo activation in the peripheral lymphoid organs, such as the spleen, and subsequently travel to the tumour site. Therefore, they encounter high adenosine concentrations in the TME after they are activated. This is an important point to consider with these results, since CD8+ T cells in this experiment were cultured in the presence of NECA from a naïve state onwards. Thus, it was important to assess how NECA affects the proliferation of activated CD8+ T cells.

Figure 3.1- The effect of NECA on the proliferation of CD8+ T Cells

(A) MACS-purified, CTV-labelled naïve CL4 CD8+ T cells were primed using anti-CD3 and anti-CD28 mAbs. NECA was added at different concentrations (1 µM, 2 µM, 5 µM, 10 µM, 20 µM or 50 µM) at 0 hours and cultures were incubated for 72 hours, whilst cells were harvested every 24 hours.

(B), (C) and (D) show 24-hour, 48-hour, 72-hour time points in more detail, respectively.

Error bars represent mean ±SEM. Data is representative of 2 separate experiments using 3 technical repeats in each experiment. Data was statistically analysed using two-way ANOVA followed by Bonferroni multiple comparison test.

ns = not significant ($p \geq 0.05$)

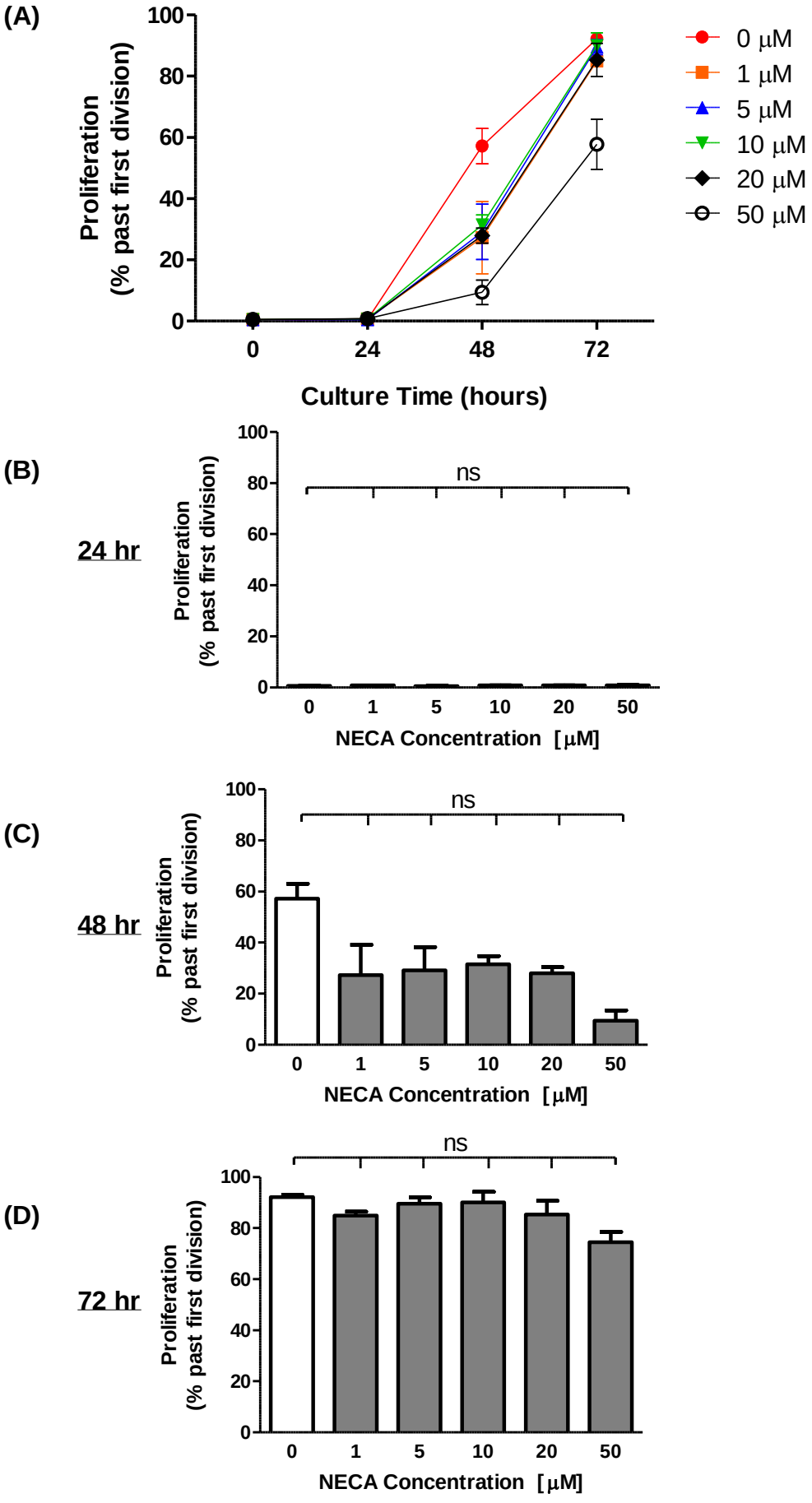

Figure 3.1 - The effect of NECA on the proliferation of CD8+ T Tells

3.3.2 At what time point during priming does NECA affect proliferation?

After encountering APCs presenting tumour antigen and undergoing activation in tumour-draining lymph nodes, CTLs enter the TME. After being subjected to the inhibitory environment CTLs become exhausted and thus tumour-tolerant, losing their ability to perform effector functions. Since cells typically enter the TME and thus encounter high adenosine levels once they are activated, NECA was added to cell culture at different points during activation to mimic this. Hence, to determine whether addition time of NECA influenced its effect on CD8+ cell proliferation, naïve CD8+ T cells were purified using MACS, labelled with CTV and primed using anti-CD3 and anti-CD28 mAbs. NECA was then added to the culture at either 0 hours, 24 hours or 48 hours of culture and cells were harvested at 72 hours (Figure 3.2).

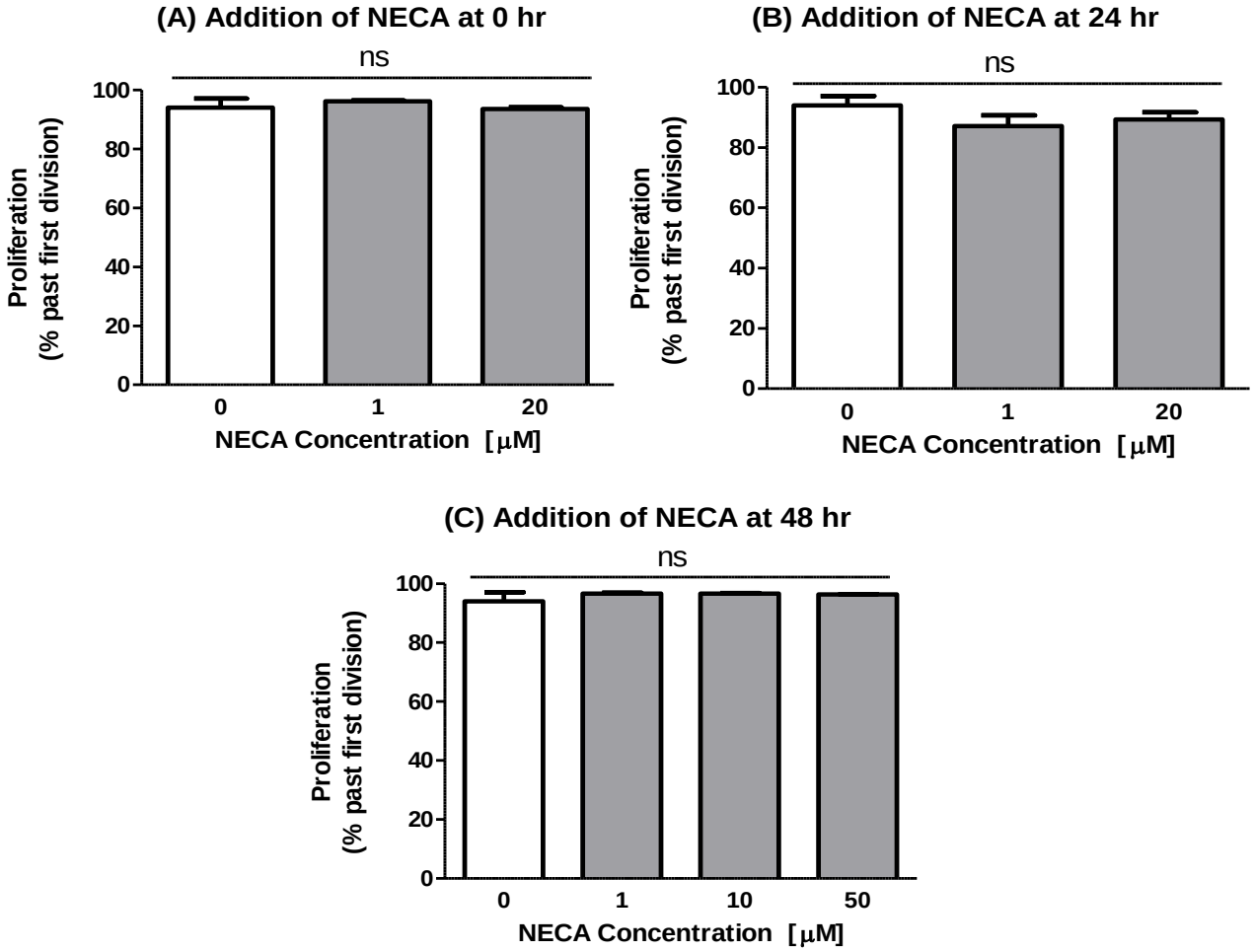

Figure 3.2 – At what time point during priming does NECA affect proliferation?

MACS-purified, CTV-labelled naïve CL4 CD8+ T cells were cultured in the presence of anti-CD3 and anti-CD28 mAbs. NECA was added at different concentrations as shown. Cells were harvested at 72 hours.

Error bars represent mean ±SEM. Data is representative of 3 separate experiments using 3 technical repeats in each experiment. Statistical analyses were carried out using one-way ANOVA followed by Bonferroni multiple comparison test.
ns = not significant ($p \geq 0.05$)

One-way ANOVA determined the differences between NECA concentrations not statistically significant when added at 0 hours ($p \geq 0.05$). This concurs with our initial findings; when NECA is when cells are still naïve (A), at 72 hours there is no difference between NECA-treated and untreated cells. However, this was also observed when NECA was added at 24 hours (B), as cells were just beginning to undergo activation. CD8+ T cells cultured in the presence of 1 µM or 20 µM NECA from 24 hours onwards had proliferated to the same extent as untreated cells by 72 hours ($p \geq 0.05$). Also when NECA was added at 48 hours (C), when cells were sufficiently activated, by 72 hours there was no significant difference between the proliferation of NECA-treated and of untreated CD8+ T cells ($p \geq 0.05$). Thus, unexpectedly the time of NECA addition appeared to have no effect on CD8+ cell proliferation.

3.4 Does NECA affect the expression of coinhibitory receptors on CD8+ T cells?

Due to the immunosuppressive nature of adenosine, it was necessary to assess how it affected naïve and activated CD8+ T cells and influenced them towards an exhausted phenotype. It is crucial to understand the interplay of immunosuppressive factors present in the TME to be able to develop effective therapies targeting them. Based on the interference of adenosine with CTL effector functions, we examined whether there is a relationship between high levels of adenosine and the expression of CIRs on the CTL surface.

3.4.1 The effect of priming on coinhibitory receptor expression on naïve CD8+ T cells

Coinhibitory receptors are upregulated on the CD8+ T cell surface not only when they are exhausted, but their expression also changes during activation. Hence, in addition to investigating how NECA affects coinhibitory receptor expression on CD8+ T cells, how the expression of these markers changed as CD8+ T cells activate was assessed. Thus, to assess the expression of TIM3, TIGIT, LAG3 and PD1 on CD8+ T cells during activation and whether this varied depending on their environment during activation, cells were primed using two different methods. Naïve CL4 CD8+ T cells were labelled with CTV and primed using either K^dHA peptide-pulsed splenocyte reaction (PSR) or by using plate-bound anti-CD3 and soluble anti-CD28 mAbs. Cells were then stained at 24-hour intervals over 96 hours with specific fluorochrome-labelled mAbs (Figure 3.3).

One-way ANOVA found no significant differences between the changes of TIM3 expression (A) when cells were activated using monoclonal anti-CD3 and anti-CD28 mAbs ($p \geq 0.05$). However, in PSR, differences in TIM3 expression were found to be significant ($p \leq 0.001$). Between 24- and 48-hour time points TIM3 rose from 10% to approximately 30%, i.e. when cells are undergoing

rapid activation. Subsequently expression plateaued, with no significant differences in TIM3 expression between 48, 72 and 96 hours.

There was a statistically significant difference between TIGIT expression changes (B) when cells were activated by anti-CD3 and anti-CD28 mAbs ($p \leq 0.01$), also when activated using PSR ($p \leq 0.01$). When cells were primed using anti-CD3 and anti-CD28 mAbs, there was a significant increase in TIGIT expression between 48-hour and 72-hour time points. Then, after 72 hours TIGIT expression had reached a maximum of approximately 40% which stayed the same at 96 hours with no significant difference. During PSR priming no significant changes between subsequent time points were observed, apart from an overall increase by 30% between 0 hours and 48 hours, until TIGIT expression dropped off steeply from 35% to 10% between 72 and 96 hours of culture.

Analyses of differences in LAG3 expression (C) found statistically significant differences when cells were activated using anti-CD3 and anti-CD28 mAbs ($p \leq 0.01$) as well as when cells were primed using PSR ($p \leq 0.001$). Following activation using anti-CD3 and anti-CD28 mAbs, LAG3 expression increased after 24 hours and plateaued at approximately 40% over the subsequent time points. When CD8+ T cells were primed using PSR, LAG3 levels rose to 55% percentage between 0-hour and 24-hour time points and peaked at 48 hours at ~85%. Following this, at 72 hours expression had decreased to 30% percentage but a rise of 10% was seen again at 96 hours.

Differences in PD1 expression (D) were found to be statistically significant when CD8+ T cells were activated using anti-CD3 and anti-CD28 mAbs ($p \leq 0.001$). PD1 expression increased steadily until it peaked at 38% at 72 hours. Following this, there was a 20% decrease between the 72-hour and 96-hour time point. Changes in PD1 expression changes were also significant when cells were primed using PSR ($p < 0.05$). PD1 was upregulated to 35% at 24 hours when cells were beginning to activate, however subsequently expression decreased to ~10% and stayed at this level for the 72- and 96-hour time points.

Whilst increases of CIRs are seen when cells are primed by either method, it appears that in the setting of a PSR coinhibitory receptors, especially TIM3, TIGIT and PD1, are downregulated after activation (by 72 and 96 hours). This stands in contrast with the levelling of CIR expression observed in anti-CD3 and anti-CD28 priming, despite this plateau occurring at different expression levels of the CIRs. This could be due to the mixture of splenocytes present in this K^dHA peptide-pulsed (PSR) culture method, such as dendritic cells or macrophages, influencing CD8+ cells to downregulate the suppressive surface markers.

Figure 3.3 - The changes in expression of coinhibitory receptors on CD8+ T cells during activation

Naïve CL4 CD8+ T cells were primed *in vitro* using either a K^dHA peptide-pulsed splenocyte reaction (PSR) or by culturing with anti-CD3 and anti-CD28 mAbs. For PSR, cells were labelled with CTV and then plated, whilst cells activated with antibodies were MACS-purified first. Cultures were incubated up to 96 hours, with cells being harvested every 24 hours and prepared for flow cytometry analysis using fluorochrome-labelled mAbs specific for (A) TIM3, (B) TIGIT, (C) LAG3 or (D) PD1.

Error bars represent mean ±SEM. Data is representative of 3 separate experiments using 3 technical repeats in each experiment. Statistical analysis was carried out using one-way ANOVA and subsequent Bonferroni multiple comparison test.

* $p<0.05$
** $p\leq0.01$
*** $p\leq0.001$

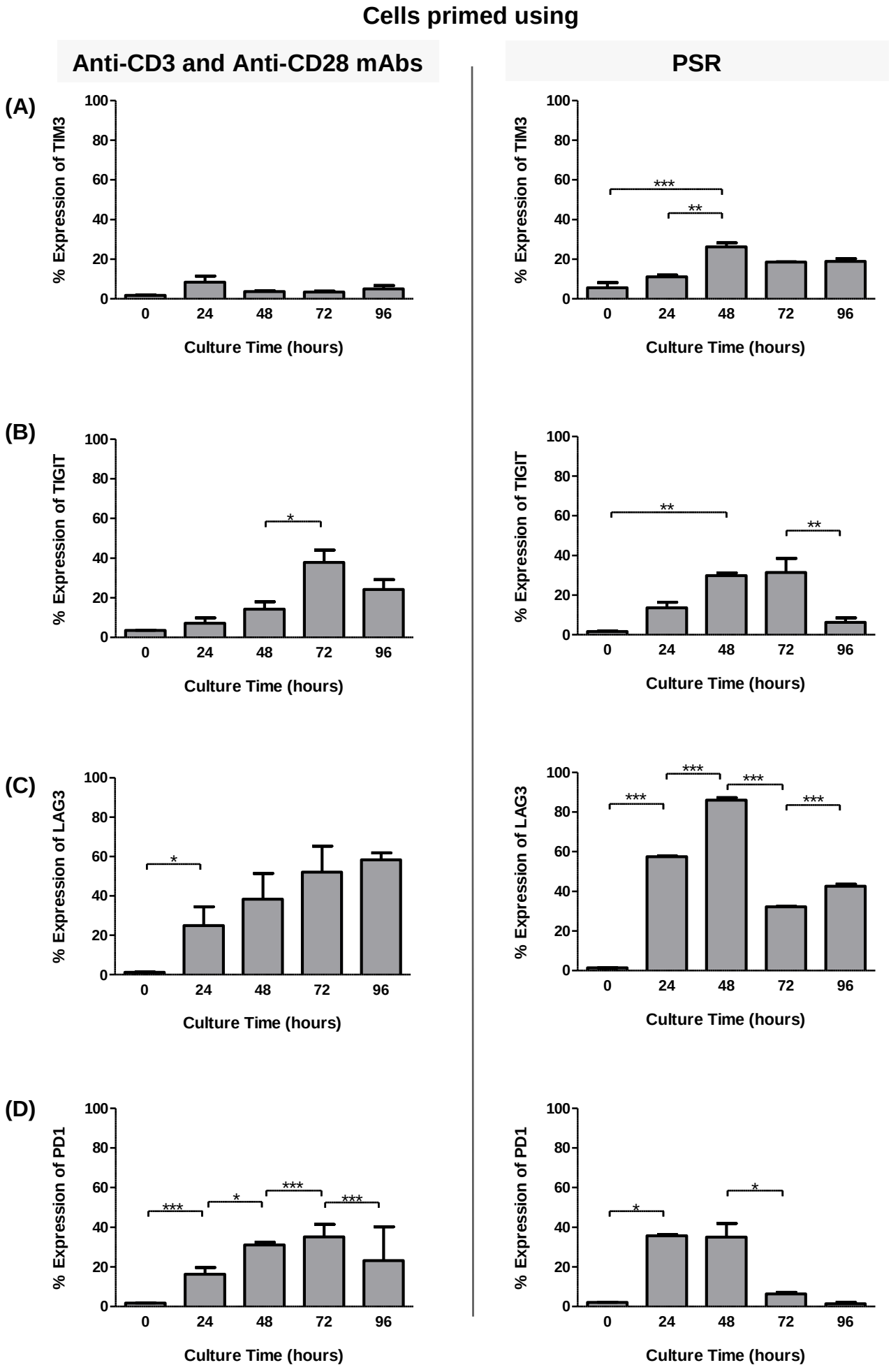

Figure 3.3 - The changes in expression of coinhibitory receptors on CD8+ T cells during activation

3.4.2 Does NECA affect the expression of coinhibitory receptors on CD8+ T cells?

After establishing the expression patterns of CIRs during priming, we assessed whether this would be impacted by NECA. CTL exhaustion has been associated with elevated levels of coinhibitory receptors on the CD8+ T cell surface. Since it is likely that the exhausted phenotype is contributed to by elevated levels of adenosine in the TME, it was important to investigate how NECA affected the expression of CIRs of CD8+ T cells directly.

To determine the effect of NECA on the expression of TIM3, TIGIT, LAG3 or PD1 on CD8+ T cells as they activate, naïve CL4 CD8+ T cells were purified using MACS and labelled with CTV. These were then primed using anti-CD3 and anti-CD28 mAbs, at which point NECA was added at 1 µM, 2 µM, 5 µM, 10 µM, 20 µM or 50 µM). Cells were cultured for 72 hours, with harvest and staining using specific mAbs occurring every 24 hours (Figure 3.4).

Analysis of the data revealed no statistically significant difference between different concentrations of NECA at different time points on the expression of TIM3 ($p \geq 0.05$) or the expression of TIGIT ($p \geq 0.05$) as determined by two-way ANOVA. The varying NECA concentrations had no effect on TIM3 or TIGIT expression and both receptors were expressed at a low level (between 10-20% expression) throughout culture time in NECA-treated and untreated wells. Further, two-way ANOVA found a statistically significant effect of different NECA concentrations at different time points on the expression of LAG3 ($p \leq 0.001$) and on the expression of PD1 ($p \leq 0.001$). LAG3 levels decreased as NECA concentration increased at 24 hours (B). 50 µM had the biggest effect on LAG3 expression, with higher NECA concentrations dampening LAG3 expression most at 24 hours and 48 hours. NECA concentrations 1 µM, 10 µM, 20 µM and 50 µM significantly decreased LAG3 expression at 48 hours (C). However, by 72 hours, this effect was reversed as LAG3 levels on NECA-treated and on untreated cells showed no significant difference.

A similar pattern could be observed for PD1 expression. Higher NECA concentrations (10 µM, 20 µM and 50 µM) significantly lowered PD1 expression from 55% in untreated cells to expression less than 30% after 24 and from 80% in untreated cells to less than 65% after 48 hours of culture, as shown in (B) and (C), respectively. At 72 hours, again there was no significant difference between untreated and treated cells (D). 1 µM and 5 µM concentrations had no effect on the expression pattern of PD1 at any time point.

Figure 3.4 - The effect of NECA on the expression of coinhibitory receptors on CD8+ T cells

(A) MACS-purified, CTV-labelled naïve CL4 CD8+ T cells were primed using anti-CD3 and anti-CD28 mAbs. NECA was added at different concentrations (1 µM, 2 µM, 5 µM, 10 µM, 20 µM or 50 µM) at 0 hours and cultures were incubated for 72 hours. Cells were harvested and stained using fluorochrome-labelled mAbs specific for TIM3, TIGIT, LAG3 or PD1 every 24 hours.

(B), (C) and (D) show 24-hour, 48-hour, 72-hour time points in more detail, respectively.

Error bars represent mean ±SEM. Data is representative of 2 separate experiments using 3 technical repeats in each experiment. Data was statistically analysed using two-way ANOVA followed by Bonferroni multiple comparison test where appropriate.

* $p<0.05$
** $p\leq0.01$
*** $p\leq0.001$
ns = not significant ($p\geq0.05$)

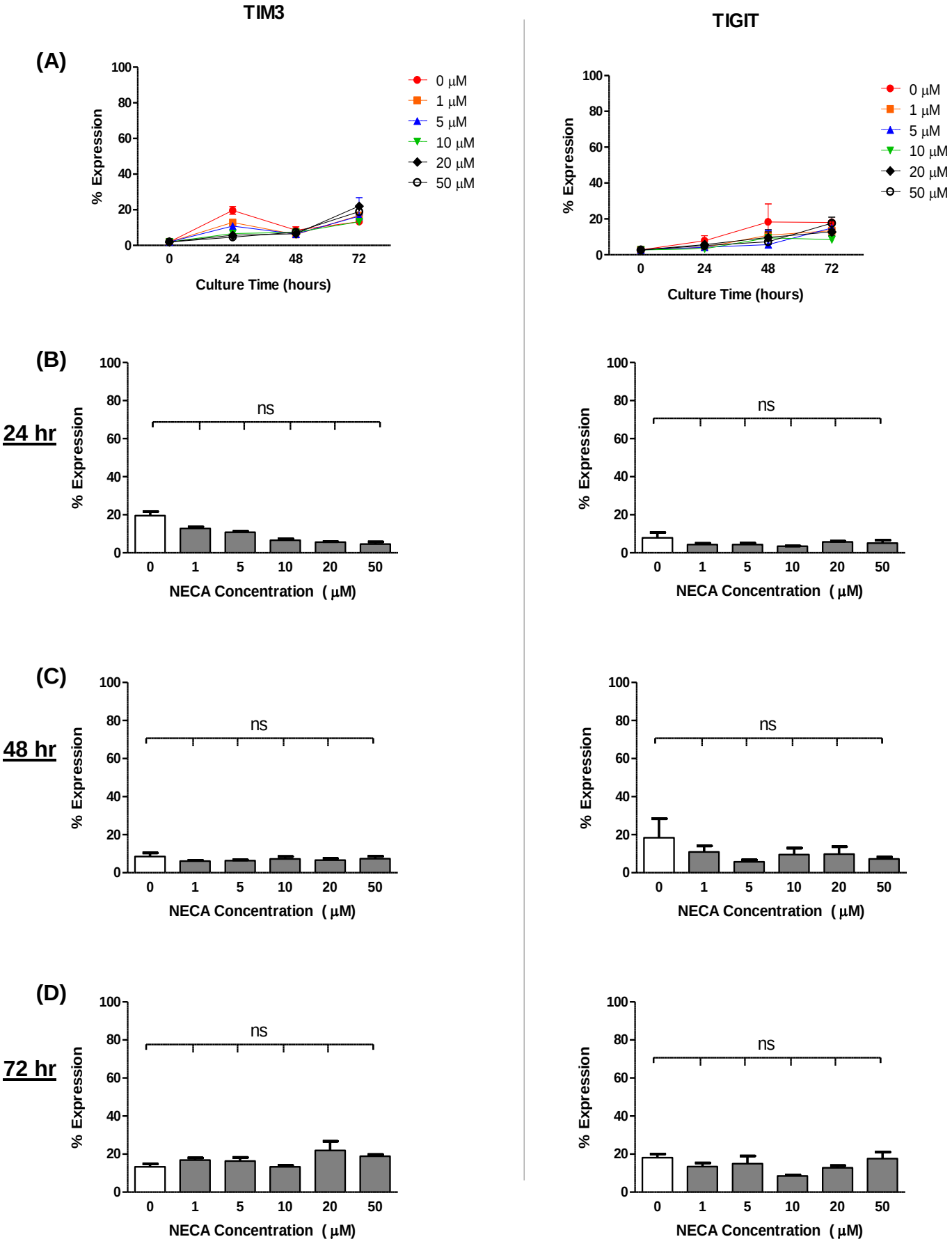

Figure 3.4 - The effect of NECA on the expression of coinhibitory receptors on CD8+ T cells

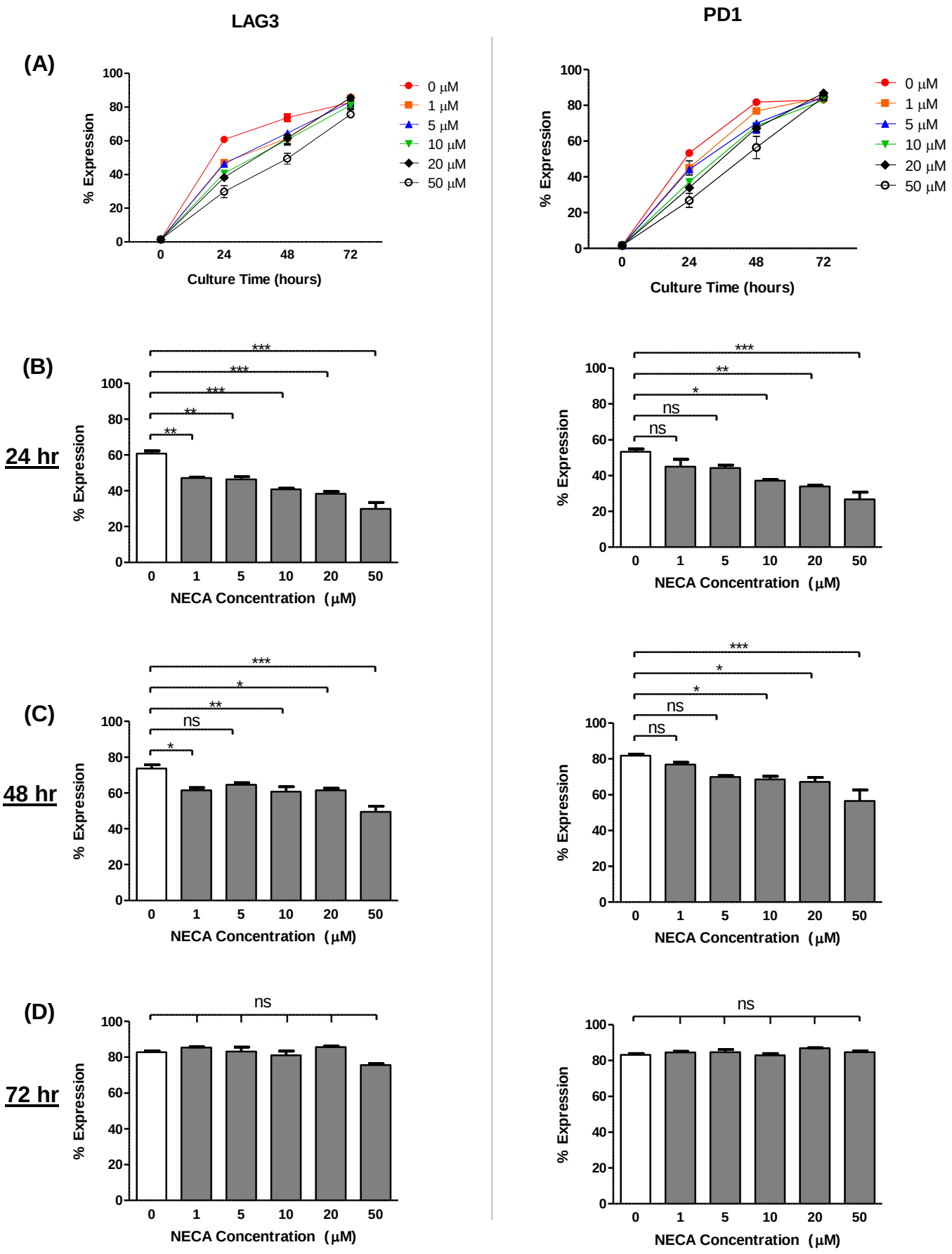

Figure 3.4 - The effect of NECA on the expression of coinhibitory receptors on CD8+ T cells

3.5 Discussion

The combination of several immunosuppressive and inhibitory factors present in the TME, such as high levels of extracellular adenosine in the tumour surroundings, drive anti-tumour CD8+ cells towards a tumour-tolerant and exhausted phenotype, slowing continued tumour growth. This exhausted state is hallmarked by a loss of effector functions, such as proliferation and production of effector cytokines IL-2 and IFN-γ, and the upregulation of coinhibitory receptors on the surface of CD8+ T cells residing in the tumour. Under normal conditions, these receptors help regulate T cell activity to prevent overactivity, however in a tumour setting this brake is taken advantage of by preventing the killing of malignant cells.

Here we assessed the effects of NECA on different aspects of CD8+ T cells, such as their proliferative ability and the expression of CIRs on their cell surface. Although CIRs are often described as being upregulated during CD8+ T cell activation, the exact trends of their expression are not described in detail. The prediction of biomarkers is essential in immunotherapy; furthering the understanding of CIR expression patterns would allow for targeting of therapies to them.

We showed the patterns of TIM3, TIGIT, LAG3 and PD1 expression during CD8+ T cell activation and found that in a mixture of splenic cells in culture CIR expression was downregulated after activation. This pattern was not observed when CD8+ T cells alone were activated via stimulation of CD3 and CD28 only, confirming that the surrounding immune cells influence CD8+ T cells and encourage their functionality. Additionally, our findings suggested that NECA has no effect on either the expression of these markers of exhaustion or on CD8+ T cell proliferation, even at concentrations higher than those encountered in the TME. These were unexpected findings, since adenosine is known to have an immunosuppressive effect on T cells (43, 48).

In the TME, adenosine very likely acts in concert with other immunosuppressive factors and its effects on coinhibitory receptors may therefore be mediated in a secondary manner rather than affecting CTLs directly. Further, it is also possible that these two inhibitory mechanisms do not interplay but act separately on CTLs instead. This premise is supported by studies in which tumour metastases were inhibited using a combination of adenosine receptor as well as CIR blockade (127). High concentrations of adenosine (typically higher than 50 μM) have been shown to act via the A2A receptor to suppress some T cell functions (128, 129), whilst some studies have found that the A3 adenosine receptor may also be involved in the suppression of CD8+ T cell activity at lower adenosine concentrations (43). In this case, these effects would not be mimicked using NECA since it is an A1 and A2 agonist. Thus, it should be investigated whether stimulation of A3 affects proliferation or CIR expression on CD8+ T cells. Further,

expression of CIRs increases in the presence of their ligands, which are upregulated on tumour and other immune cells. Thus, it is likely that the lack of ligands for TIM3, TIGIT, LAG3 and PD1 in our experimental setting discouraged their upregulation despite stimulation of adenosinergic receptors on the CD8+ T cell surface.

In conclusion, we found that stimulation of A1 and A2 receptors using NECA had no effect on CD8+ T cell proliferation or expression of TIM3, TIGIT, LAG3 or PD1 even at high concentrations. With these findings in mind, it is important to establish how adenosine affects anti-tumour CD8+ T cells. Further, although its role is debated, the effect of A3 receptor engagement should be investigated. It is important to understand expression patterns of CIRs as well as mechanisms of suppression via adenosine to be able to successfully target these in cancer therapies.

Chapter 4 - Priming CD8+ T Cells using SAGEs

4.1 Background

Cancer immunotherapies are aimed at utilising and enhancing the host's own immune system. This makes immunotherapy less invasive and allows potential targeting of therapies, thus reducing side effects experienced by the patient than when compared to traditional therapies. Vaccines to help mount sufficient anti-tumour immune responses are actively being investigated, with the goal of potentiating or restoring tumour immunity. Several studies have found that the presence of high numbers of TILs in tumours indicates good prognoses in several cancers (81-85). This highlights the need of tumour vaccines to stimulate tumour-antigen-specific proliferation of CTLs to provide large numbers of these cells exhibiting effective cytotoxic functions and to establish memory T cells to provide long-term immunity. In order to achieve this, different parts of the anti-tumour immune response can be targeted. This may include DCs since these cells are considered professional APCs due to their efficiency in antigen uptake and presentation via MHC class molecules (130, 131). Immature DCs are able to capture antigen, however upon presentation of antigen to T cells no costimulatory signals are provided (130). Immature DCs are associated with the induction of tolerance and immunosuppressive characteristics (130, 132). On the other hand, mature (activated) DCs present antigen-MHC molecule complexes to the TCR and provide costimulation via B7.1 and B7.2 to activate naïve CD8+ T cells and initiate their differentiation to CTLs (132). Thus, cancer vaccines are often targeted to this step, in order to initiate proliferation of CTLs primed against tumours and gain large CTL numbers. This may be achieved by injection of DCs that have prior been pulsed with tumour-antigen in order to promote T cell immunity (133, 134).

Early attempts at tumour vaccinations used short peptides, often without using an effective adjuvant, to activate DCs which did not prove promising. This was likely due to particles being cleared before DCs had the opportunity to internalise them and the lack of activation triggered a steady state in DCs. Adding immune stimulants into the equation has been shown to increase the effectiveness of cancer vaccines (15). However, as knowledge of tumour immunology advances, more hurdles are identified in the development of effective cancer vaccines. Firstly, the tumour antigen to be used must be considered. Ideally, this antigen is expressed highly on tumours in many patients with the same cancer whilst found not at all or only low levels on healthy tissue. In addition, the ideal tumour antigen is vital for the tumour's growth or survival (to reduce chances of immune evasion) (15, 109). Another difficulty in developing a potent and effective cancer vaccine lies in that the vaccine needs to break the tumour-induced tolerance of anti-tumour immune cells successfully. In order to do so, the vaccine must deliver large

quantities of antigen to DCs, stimulate their expansion and provide DC activation signals (109). Subsequently, activated DCs should migrate to the lymph nodes to present the antigen to Cd8+ T cells to then activate these strongly to ensure high TCR affinity and effective cytotoxic functions (15, 109).

Vaccination of free peptide with adjuvant can counterproductively lead to tolerance of CD8+ T cells. This is likely due to the distribution of peptide systemically causing overstimulation and subsequent cell death of specific CD8+ cells. Thus, if the peptide is delivered to the target in a contained manner, this improves the cytotoxic cell response (135).

SAGE particles are synthetic nanoparticles consisting of short, *de novo* α-helical peptides. Self-assembly out of two complementary hubs (a heterodimer and a homotrimer) creates closed networks that allow the attachment or enclosure of material (117). Due to the interchangeability of their components, SAGEs and their potential applications can be highly versatile, such as the delivery of drugs or biomolecules (115, 117). By attaching tumour antigens to SAGEs, these could be used as tumour vaccines. Recent studies using SAGEs have shown their immunogenic potential; SAGEs can be targeted to and be taken up by APCs *in vitro.* Further, SAGEs elicited antigen-specific proliferative responses from CD4+ and B cells *in vivo* (116).

In this study, we tested the ability of peptide-conjugated SAGEs to activate effector functions of CD8+ T cells *in vivo*. Functionalisation of the particles with peptides specific to the CL4 TCR allowed the targeting to specific CD8+ T cells. For this, three different-length peptides were used. Epitope (EP) peptide (H-IYSTVASSL-OH) was synthesised based on K^dHA peptide (^{518}IYSTVASSL^{526}) serving as a specific peptide to be recognised by the TCR expressed on CL4 CD8+ T cells. Further, POL (H-AVGAGATAEEIYSTVASSL-OH) and Leader (LEA) peptides (H-EKLAGFGAVGAGATAEEIYSTVASSL-OH) were used. De Haan *et al* (2002) used a loop segment derived from the DNA polymerase of the herpes simplex virus type 1 to add to a subunit of the *Escherichia coli* heat-labile toxin in order to use this subunit as an epitope-delivery vehicle for MHC class I presentation (136). Based on this work, the mentioned loop segment containing hydrophobic, charged amino acids was added to the epitope sequence (EP) to give the POL peptide. This was done in order to be able to target the SAGE-delivered peptide for internalisation by APCs and into the cross-presentation pathway *in vivo*. Further, in addition to the loop segment, a cathepsin cleavage site was added. This formed the LEA peptide which was created to potentially improve uptake of the peptide by APCs in case the POL peptide would not elicit sufficient uptake by APCs.

4.2 Aims

I. To evaluate the ability of EP, POL and LEA peptides to activate CD8+ T cells

II. To evaluate the ability of POL-SAGEs to prime CD8+ T cells *in vivo*
III. To establish whether POL-SAGEs can improve CD8+ T cell proliferation compared to POL peptide alone

4.3 Testing EP, POL and LEA peptides for their ability to prime CD8+ T cells

In order to determine the ability of EP, POL and LEA peptide to activate CD8+ T cells, Renca cells were pulsed with each of the peptides. Following this, Renca cells were co-cultured with CD8+ T cells that had previously been purified using MACS and labelled with CTV. Cultures were incubated for 72 hours, whilst cells were harvested and stained for flow cytometry using specific mAbs every 24 hours (Figure 4.1). One-way ANOVA found a significant difference in levels of activation over time for each peptide ($p \leq 0.001$). In general, proliferation of CD8+ T cells in EP-pulsed wells was significantly lower than the proliferation observed in wells with the longer peptides POL or LEA. EP and POL peptides did not activate CD8+ T cells as well as the positive control, but the proliferation of cells observed in wells with LEA showed no difference to that of the positive control. CTV histograms between wells with positive control and with LEA-pulsed Renca cells are near identical, as shown in (C). Furthermore, CD8+ T cells cultured with POL- and LEA-pulsed Renca cells were highly divided, with LEA exhibiting the most divided cells. These results were surprising since CD8+ T cells cocultured with peptide-pulsed Renca cells had proliferated regardless of which peptide had been added. A likely explanation is that the irradiation of Renca cells was incomplete or did not affect the processing ability, so that Renca cells were capable of taking up the peptides, processing and displaying them to CD8+ cells. This concurs with LEA causing the highest proliferation of all three peptides since it is the longest one and thus would be more likely to be processed.

To be able to assess whether EP, POL and LEA are able to prime CD8+ T cells without potential processing of the peptides, the experiment was altered to use P815 mastocytoma cells instead of irradiated Renca cells. These cells possess MHC class I molecules to present the peptides to CD8+ cells (137). Thus, MACS-purified CD8+ T cells were labelled with CTV and co-cultured with EP-, POL- or LEA-pulsed P815 cells. Cells were harvested and stained using specific fluorochrome-labelled mAbs for flow cytometry every 24 hours over a total culture time of 72 hours (Figure 4.2). As the time course (A) shows, the percentages of cells divided were similar between EP, POL and LEA at each time point. Initially there were no divided cells at 24 hours which changed by 48 hours as a steep rise could be seen when cells activated. At 72 hours there were no significant differences in percentage of cells proliferated past the first division between the peptides. As shown in the histogram (B), successive CTV peaks of cells cultured in the presence of LEA- or POL-pulsed P815 cells are higher than those with EP-pulsed P815

cells. This suggests that LEA- or POL-pulsed P815 cells elicited more highly divided CD8+ T cells than EP-pulsed P815 cells, concurring with observations described in Figure 4.1. It seems unlikely that the longer peptides, especially LEA, were able to be directly presented by MHC class I molecule and thus be recognised by TCR since the optimal peptide length for the TCR to recognise is between 8 - 10 amino acid residues (138, 139). Whilst longer peptides may still be recognised, it would not be anticipated that subsequent CD8+ T cell activation would be of the same magnitude as one from a much shorter peptide (140). Thus, these observations suggest degradation of the longer peptides (into EP) or the presence of excess peptide in solution.

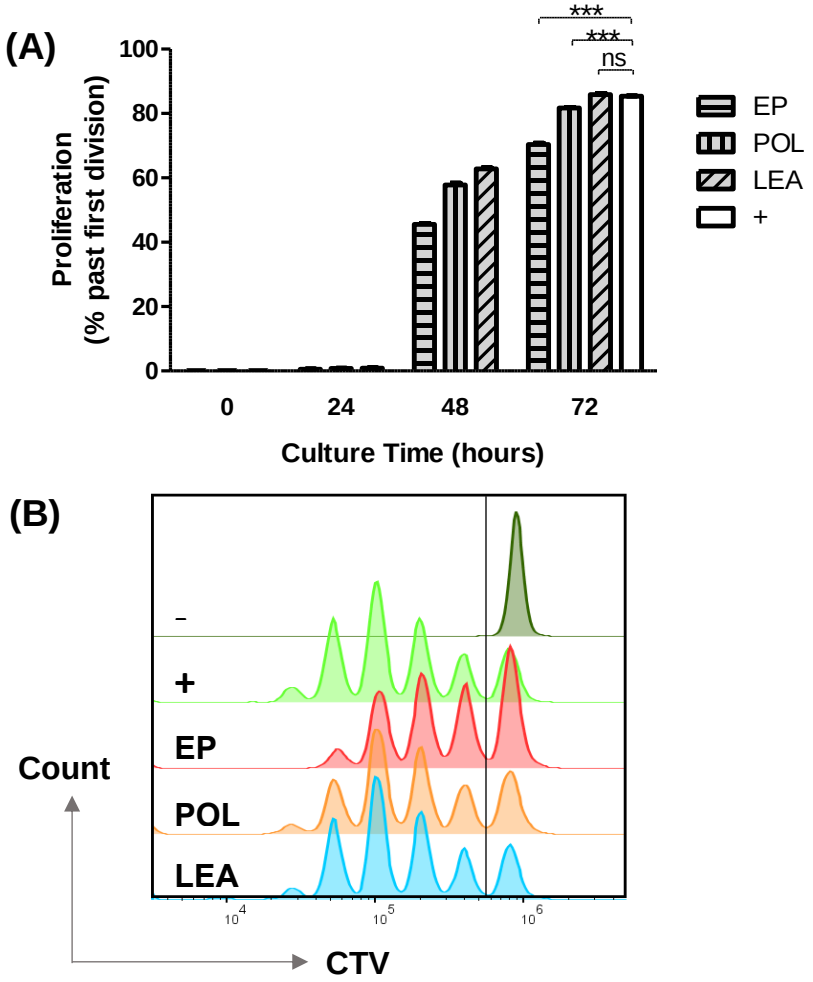

Figure 4.1 - Testing EP, POL and LEA peptides using Renca cells

(A) MACS-purified, CTV-labelled naïve CL4 CD8+ T cells were co-cultured with irradiated Renca cells which were peptide-pulsed with either EP, POL or LEA peptide at 1 mM or K^dHA (positive control) or unpulsed (negative control). Cultures were incubated for 72 hours; cells were harvested and stained using fluorochrome-labelled mAbs specific for CD8α every 24 hours.

(B) Histogram shows CTV profiles at 72 hours for each peptide. Gates are representative of percentage of cells divided past the first peak. Proliferation was measured by assessing cells past the first undivided peak shown in the first row.

Error bars represent mean ±SEM. Data is representative of 3 separate experiments with 3 technical repeats each experiment. Statistical analyses were carried out using one-way ANOVA followed by unpaired t-test.

*** $p \leq 0.001$
ns = not significant ($p \geq 0.05$)

Figure 4.2 – Testing EP, POL and LEA peptides using P815 cells

(A) MACS-purified, CTV-labelled naïve CL4 CD8+ T cells were co-cultured with P815 cells which were previously mitomycin-treated and peptide-pulsed with either EP, POL or LEA peptide at 1 mM. The positive and negative control were co-cultured with K^dHA-pulsed or unpulsed P815s, respectively. Cultures were incubated for 72 hours; cells were harvested and stained using fluorochrome-labelled mAbs specific for CD8α every 24 hours.

(B) shows 48-hour and 72-hour time points in more detail.

(C) Histogram shows CTV profiles at 72 hours for each peptide. Gates are representative of percentage of cells divided past the first peak. Proliferation was measured by assessing the percentage of cells divided past the first peak, as shown in Figure 6.4 in the appendix.

Error bars represent mean ±SEM. Data is representative of 4 separate experiments with 3 technical repeats each experiment. Statistical analyses were carried out using one-way ANOVA followed by unpaired t-test.

ns = not significant ($p \geq 0.05$)

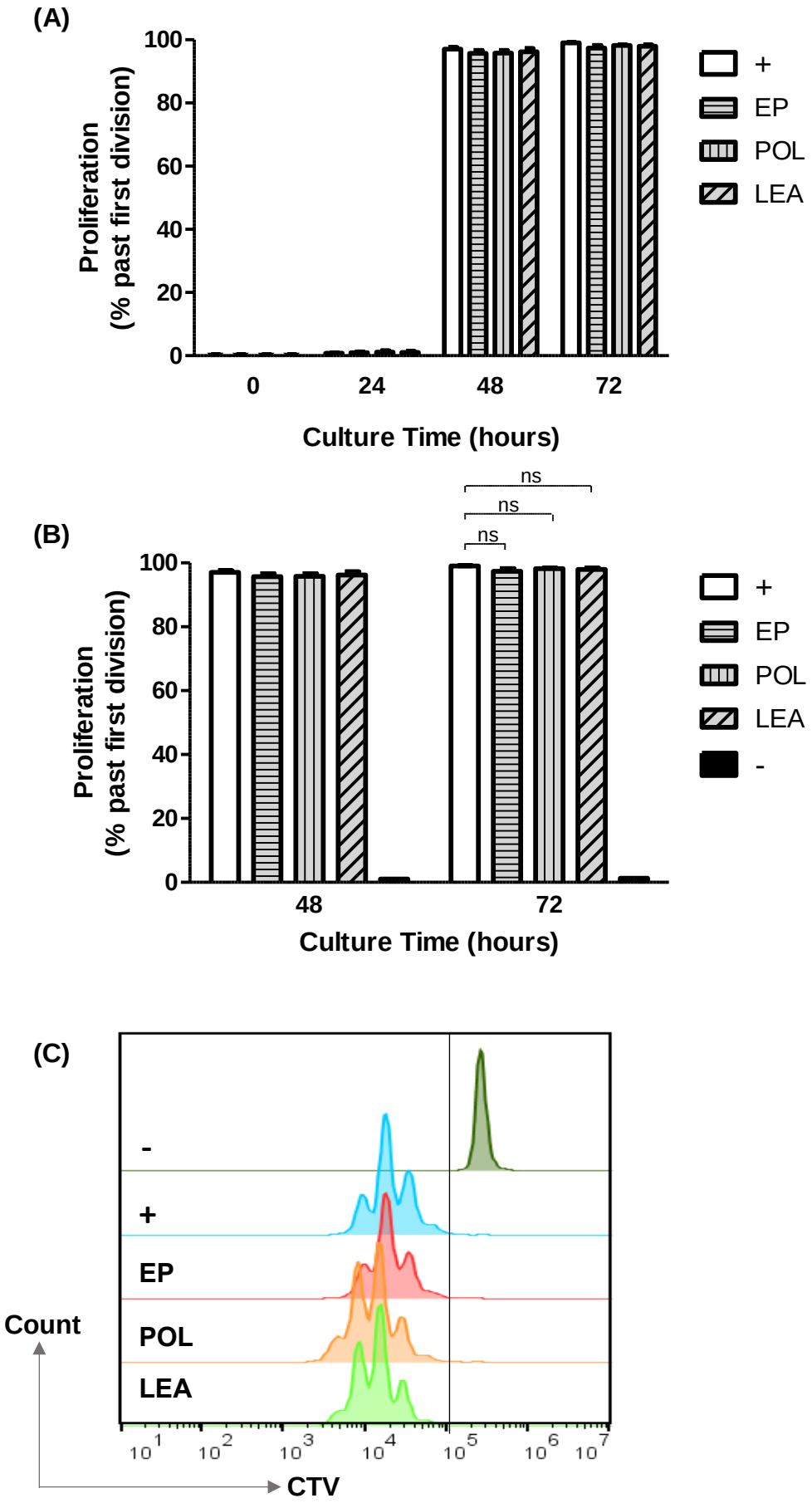

Figure 4.2 - Testing EP, POL and LEA Peptides using P815 Cells

4.4 Determining the optimal incubation time for CD8+ T cells *in vivo*

It was important to identify the optimal length of time to incubate adoptively transferred CL4 CD8+ T cells to use in our investigations. Thus, in order to optimise the time period for which CD8+ T cells would be incubated (where the majority of cells had undergone several rounds of cell division without losing the proliferative dye completely) in *in vivo* experiments, naïve CD8+ cells were purified using MACS, labelled with CTV and adoptively transferred into BALB/c mice. Following adoptive transfer, mice were inoculated with 1200 HA units of A/PR/8/H1N1 influenza virus. Mice were culled after either 3 or 7 days and CD8+ T cells were extracted from spleens (Figure 4.3).

The data shown in Figure 4.3 shows that a higher percentage of cells had divided at day 7 than at day 3. As can be seen in (B), most cells had lost the CTV dye by day 7 which created two distinct populations, one of high CTV fluorescence and one of no CTV fluorescence. Whereas at day 3 clear populations of cells undergoing different rounds of divisions could be observed, thus this incubation time was used in subsequent experiments.

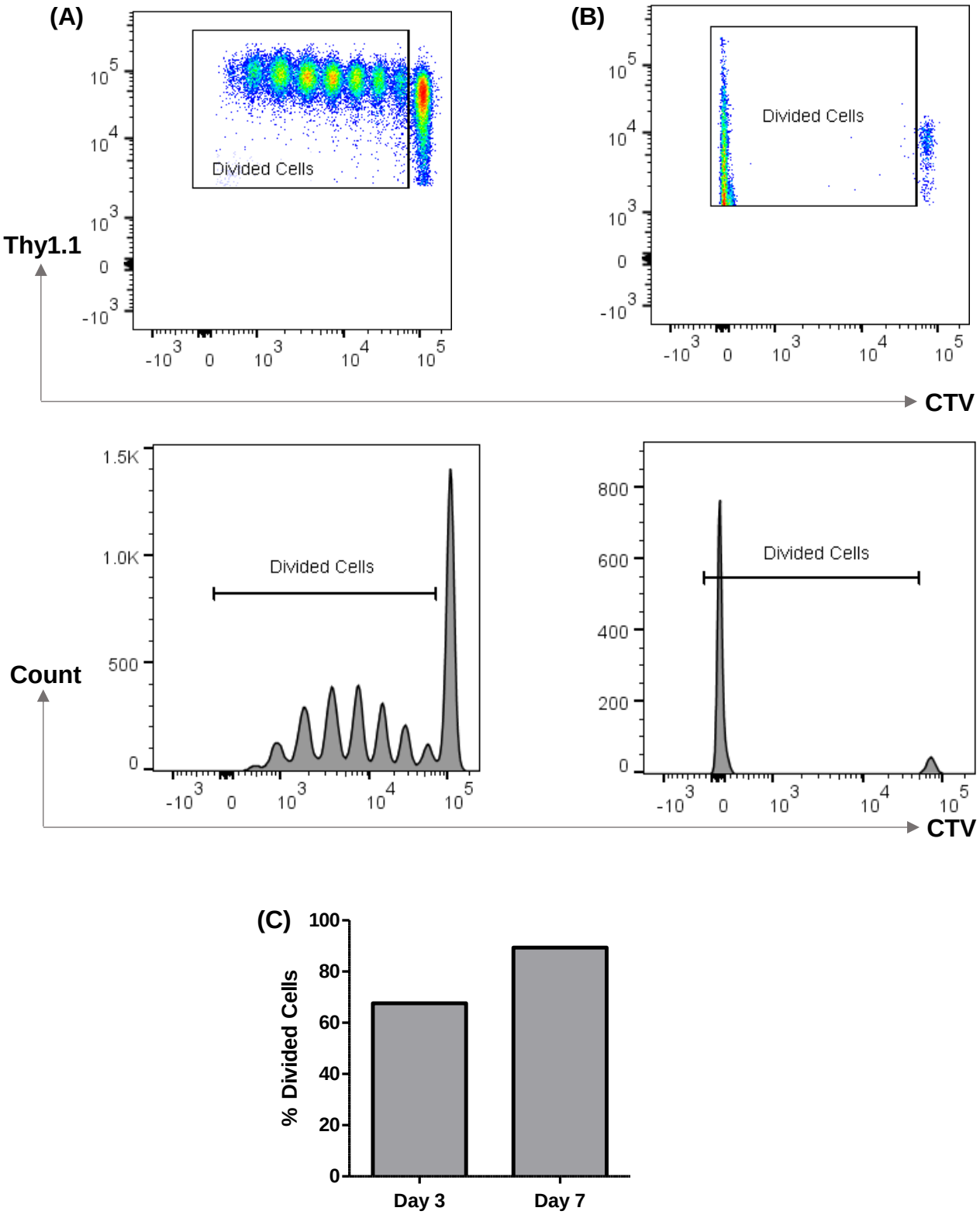

Figure 4.3 - Determining the optimal incubation time for CD8+ T cells in vivo

6- to 8-week old BALB/c mice received 3-5 x10^6 MACS-purified, CTV-labelled naïve CL4 CD8+ T cells by adoptive transfer and subsequently received approximately 1200 HA units of influenza virus strain A/PR/8/H1N1. After either (A) 3 or (B) 7 days CL4 CD8+ T cells were extracted from spleens using MACS and stained for flow cytometry.

Data shown is from one single experiment where n=1 for day 3 and n=2 for day 7.

4.5 Evaluating the ability of POL-SAGE to activate CD8+ T cells *in vivo*

Due to the similarity in size and shape of SAGE molecules to viral particles, some immunogenicity may be expected of them (116). It was important to determine whether an immune response was elicited in this experimental setting, to be able to identify false positive results.

In order to assess whether unconjugated SAGE particles would elicit a proliferative response, MACS-purified and CTV-labelled CD8+ T cells were cultured in the presence of SAGE particles for 72 hours. Cells were then harvested and stained for flow cytometry using CD8α-specific mAbs (Figure 4.4).

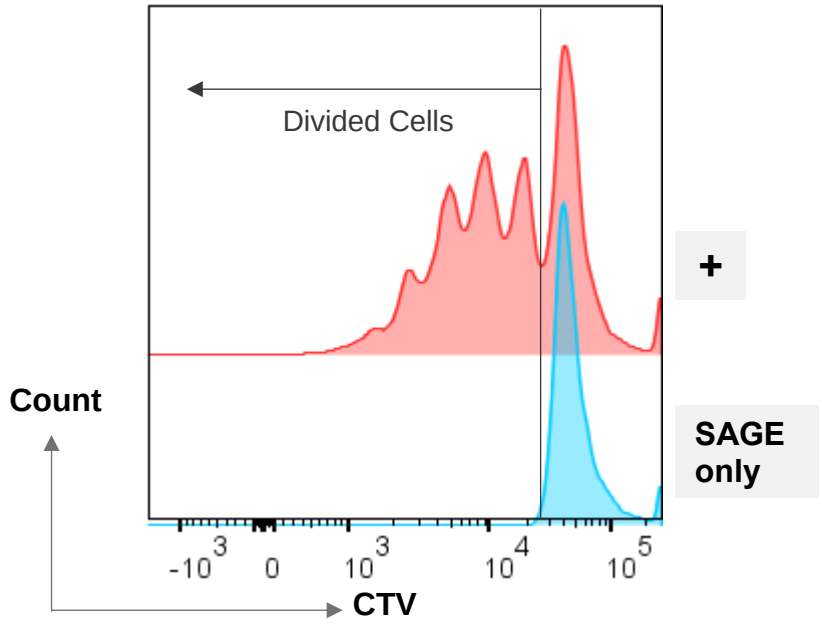

Figure 4.4 - Does unconjugated SAGE alone initiate CD8+ T Cell proliferation?

MACS-purified, CTV-labelled naïve CL4 CD8+ T cells were mixed with either unconjugated SAGE particles or K^dHA (positive control). Cultures were incubated for 72 hours; cells were harvested and stained using fluorochrome-labelled mAbs specific for CD8α after this.
Data is representative of 2 separate experiments.

We found no proliferation of CD8+ T cells cultured in the presence of SAGE particles. As can be seen in Figure 4.4, the peak seen for cells cultured with SAGE aligns with the undivided peak of cells in the positive control. Thus can be concluded that the parent SAGE particle alone cannot prime CD8+ T cells without the functionalisation with appropriate peptides.

After establishing that the EP, POL and LEA peptides were able to prime CD8+ T cells *in vitro* (Section 4.3), we wanted to test whether peptide delivery was possible using SAGEs. Thus, it was important to determine whether encapsulation and delivery of POL peptide using

conjugated POL-SAGEs would be able to prime CD8+ T cells sufficiently *in vivo* and how the immune response compared to a positive control. In order to do so, MACS-purified, CTV-labelled CD8+ cells were adoptively transferred into BALB/c mice followed by inoculation with either influenza virus or POL-SAGE. CD8+ T cells were extracted 3 days later, stained for flow cytometry using specific mAbs and analysed (Figure 4.5). As seen in (C), a higher percentage of cells had proliferated in mice with POL-SAGE treatment than in the positive control. These findings suggest that POL-SAGE was able to prime CD8+ T cells *in vivo* and drive a strong proliferative response. In order to establish whether proliferation of CD8+ cells was due to POL-SAGE particles or whether this was due to one of the SAGE constituents, adoptive transfer was performed in an identical manner as above in a separate experiment. This was followed by inoculation with either POL peptide only, unconjugated SAGE + POL peptide, or POL-SAGE (Figure 4.6). There were no divided CD8+ T cells in the negative control, whilst CD8+ T cells in the positive control had proliferated, which indicates a functioning system and that CD8+ T cells did not proliferate without external intervention. Results showed that the percentages of divided cells were very similar between the different conditions. Constructing SAGE with the POL peptide did not improve the proliferative response compared to peptide alone.

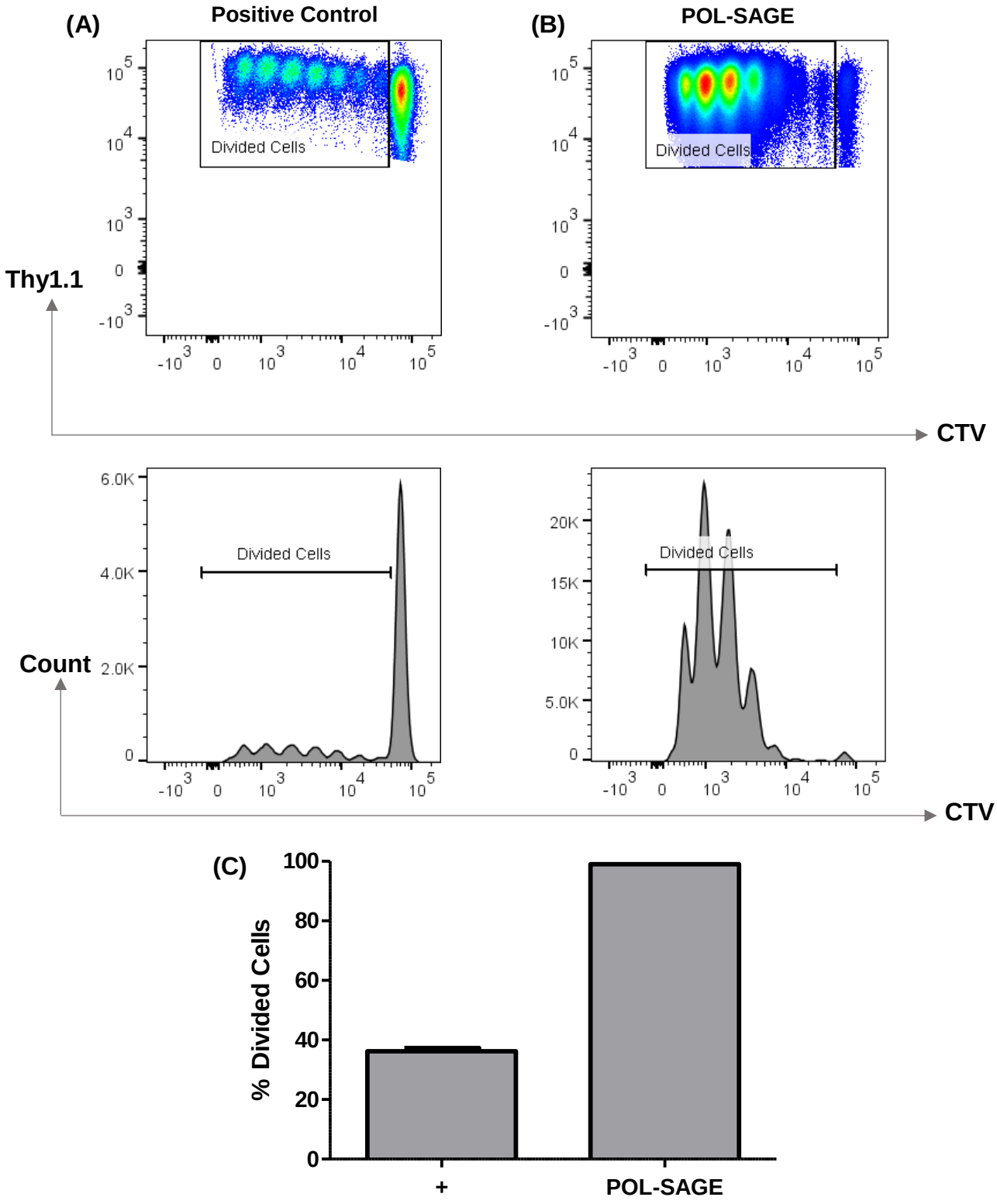

Figure 4.5 - Assessing the activation ability of POL-SAGEs *in vivo*

6- to 8-week old BALB/c mice received 3-5 x10^6 MACS-purified naïve CL4 CD8+ T cells that had been labelled with CTV by adoptive transfer followed by either appr. 1200 HA units of influenza virus strain A/PR/8/H1N1 or 1 mM of POL-SAGE. 3 days later CL4 CD8+ T cells were extracted from spleens using MACS and stained for flow cytometry. (A) and (B) show dot plots and histograms of mice injected with influenza or POL-SAGE, respectively. (C) shows a bar graph comparing the percentages of divided cells in both conditions.

Data shown is from one single experiment where n=2 for positive control and n=1 for POL-SAGE. Error bars represent mean ±SEM

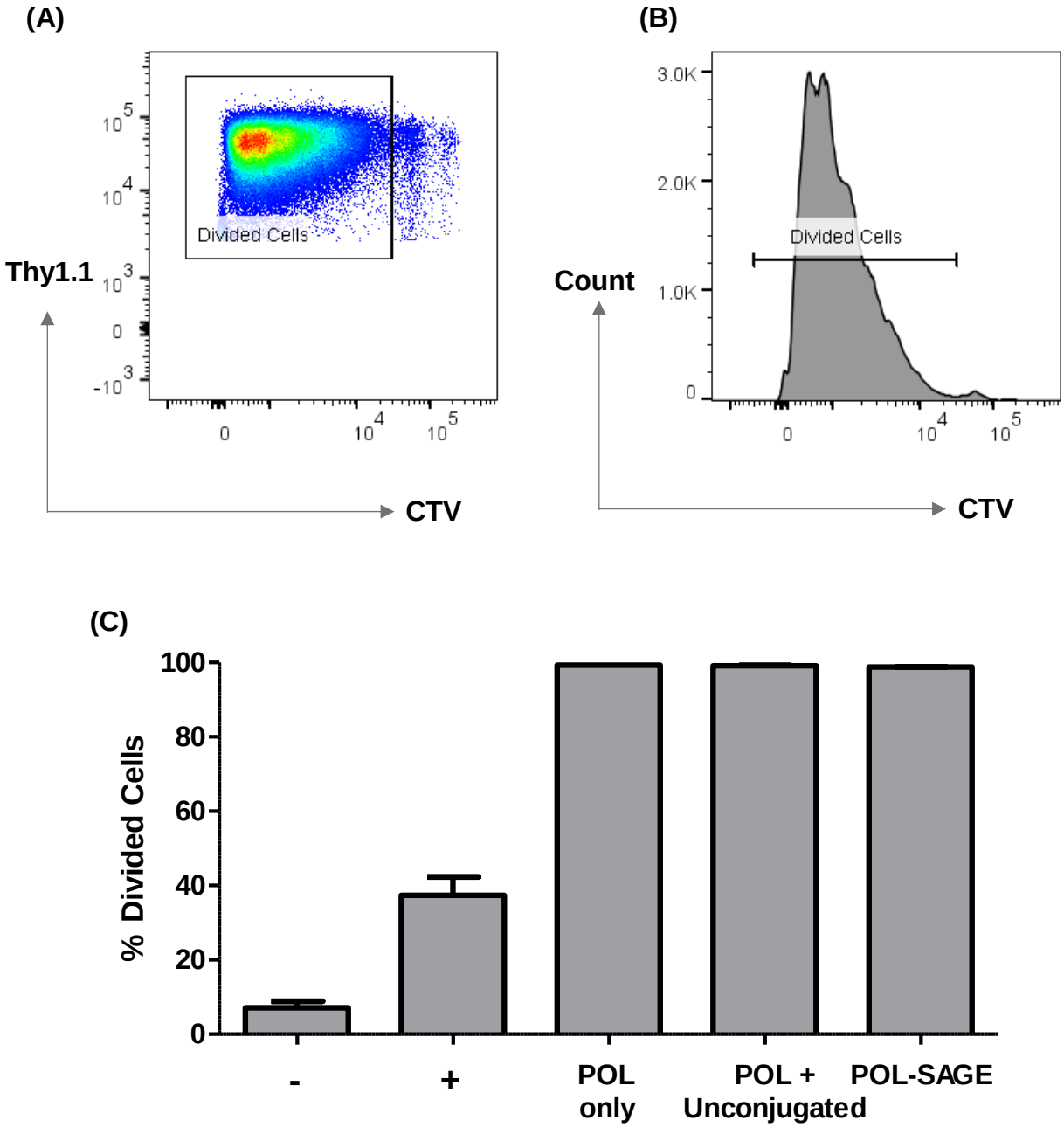

Figure 4.6 - Assessing the activation ability of POL-SAGE constituents *in vivo*

6- to 8-week old BALB/c mice received 3-5 x10^6 MACS-purified, CTV-labelled naïve CL4 CD8+ T cells by adoptive transfer followed by PBS only, appr. 10^6 EID_{50} units of influenza virus strain A/PR/8/H1N1, 1 mM of POL peptide, unconjugated SAGE Trimer particles plus POL peptide or 1 mM of POL-SAGE. 3 days later CL4 CD8+ T cells were extracted from spleens using MACS and stained for flow cytometry.

(A) shows dot plots of Thy1.1 against CTV and (B) shows the CTV histogram for the POL-SAGE sample.
(C) shows a bar graph comparing the percentages of divided cells in the different conditions.

Data shown is from one single experiment where n=2. Error bars represent mean ±SEM.

4.5.1 Do diluted EP, POL and LEA peptides activate CD8+ Cells *in vitro*?

As described in Section 4.5, both free POL peptide and POL-SAGE provoked strong proliferative responses from CD8+ T cells *in vivo*. In order to determine whether the observed proliferation was due to excess peptide in solution, we assessed whether or not the same effect is observed when EP, POL and LEA peptides were diluted *in vitro*. This was conducted by co-culturing naïve, MACS-purified CD8+ T cells that had been labelled with CTV and peptide-pulsed P815 cells (shown in Figure 4.7). The data shows that there was a statistically significant effect of the different peptides at different concentrations on the proliferation of CD8+ T cells ($p \leq 0.001$). Proliferation could be observed amongst cells cultured in the presence of POL or LEA peptides at stock concentration but as they were diluted by 10-fold, the percentage of divided cells decreased rapidly. Whilst 10^{-6} dilution of EP elicited division of only some cells, the decline in proliferation across EP concentrations was more gradual than POL or LEA dilutions and showed a clear distinction between the short peptide's ability to trigger proliferation compared to the longer ones.

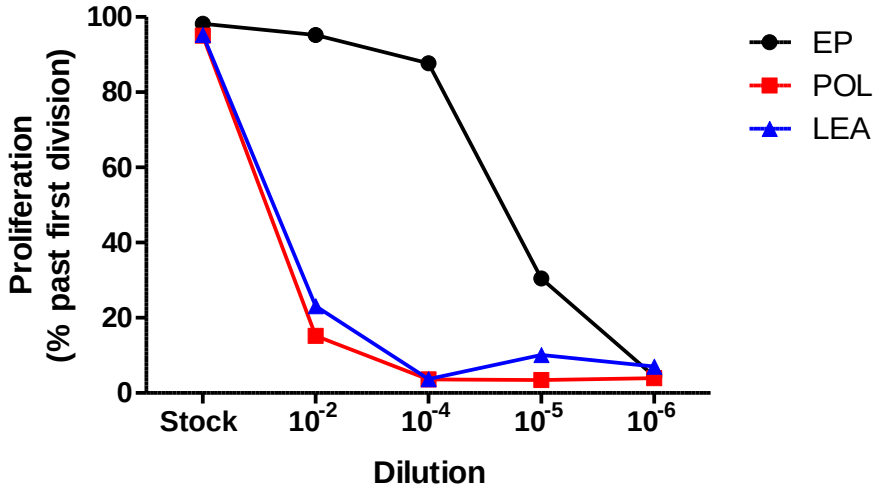

Figure 4.7 - Testing diluted EP, POL and LEA peptides for CD8+ cell activation ability *in vitro*

MACS-purified, CTV-labelled naïve CL4 CD8+ T cells were co-cultured with P815 cells which were previously mitomycin-treated and peptide-pulsed with either EP, POL or LEA peptide at different concentrations (stock: 1mM, 2-fold, 4-fold, 5-fold or 6-fold dilution of stock). Cultures were incubated for 48 hours at which point cells were harvested and stained using specific fluorochrome-labelled mAbs.

Data is representative of 2 separate experiments with 3 technical repeats each experiment. Error bars represent mean ±SEM. Statistical analyses were carried out using two-way ANOVA followed by Bonferroni multiple comparison test.

4.5.2 Can diluted POL-SAGE activate CD8+ T cells *in vivo*?

Since the data in Figure 4.7 suggested that dilution of EP, POL and LEA peptides *in vitro* affected the ensuing CD8+ cell proliferation, it was important to determine whether this held true *in vivo* and that dilution of the peptides would decrease the proliferative response observed. Thus, following adoptive transfer, mice were treated with either 1 mM or 4-fold diluted EP, POL or LEA peptide. CD8+ T cells were then extracted 3 days later, stained using fluorescently-labelled mAbs and analysed (Figure 4.8).

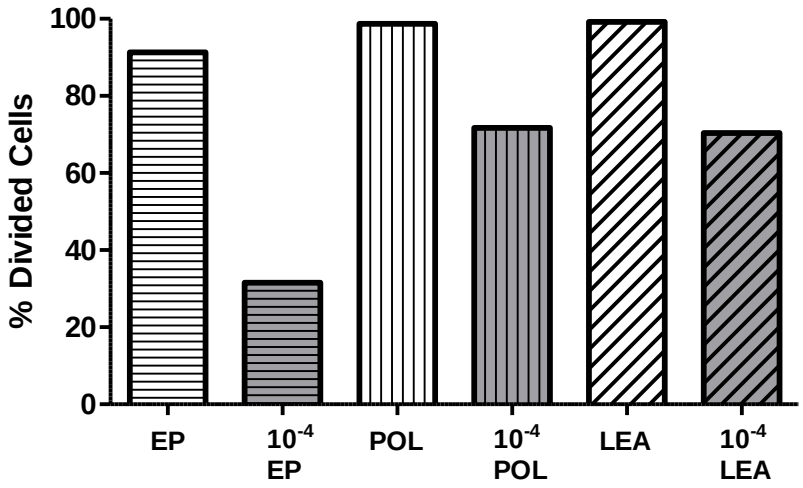

Figure 4.8 – Do diluted EP, POL and LEA peptides elicit proliferative responses *in vivo*?

6- to 8-week old BALB/c mice received 3-5 x10^6 MACS-purified, CTV-labelled naïve CL4 CD8+ T cells by adoptive transfer and subsequently received 1 mM of peptide (EP, POL or LEA) or 4-fold diluted peptide (EP, POL or LEA). 3 days later CL4 CD8+ T cells were extracted from spleens using MACS and stained for flow cytometry with fluorescently-labelled mAbs.

Data shown is from one single experiment where n=1.

The data in Figure 4.8 revealed that the percentage of divided cells decreased when peptides were diluted. Whilst undiluted EP, POL and LEA elicited similar levels of divided cells, once diluted more divided cells were seen in mice treated with diluted POL or diluted LEA peptide than in those treated with diluted EP. This finding indicates that the results from our previous *in vitro* peptide dilution (Section 4.5.1) do not directly translate to an *in vivo* setting. This is likely due to the fact that *in vivo* the peptides are internalised by APCs, such as dendritic cells, processed and displayed to CD8+ T cells via cross presentation on MHC class I and thus drive their proliferation. This system is not in place in the *in vitro* setting where the peptides are presented via the MHC class I only without processing. This concurs with the fact that once diluted, POL and LEA initiated more proliferation than diluted EP. Since APCs prefer internalising longer peptides over shorter ones (139), POL (19 residues) and LEA (26 residues) are more likely to be processed for presentation than EP (9-mer).

4.5.3 Dilution of POL peptide *in vivo*

The data in Figure 4.8 showed that dilution of EP, POL and LEA decreased the CD8+ T cell proliferative response *in vivo* compared to when using their stock concentrations. In order to establish which concentrations of POL peptide would elicit a CD8+ T cell proliferative response of appropriate extend to be used in subsequent experiments, a serial dilution of POL was conducted *in vivo* to identify the most suitable concentration for use when comparing POL peptide with POL-SAGE. BALB/c mice received an adoptive transfer of CTV-labelled, MACS-purified CD8+ cells and were subsequently treated with stock (200 µM), 10^{-2}, 10^{-3} or 10^{-4} dilutions of POL peptide. 3 days later, CD8+ cells were extracted from spleens using MACS and stained using specific fluorescently-labelled mAbs (Figure 4.9). Lower concentrations were used

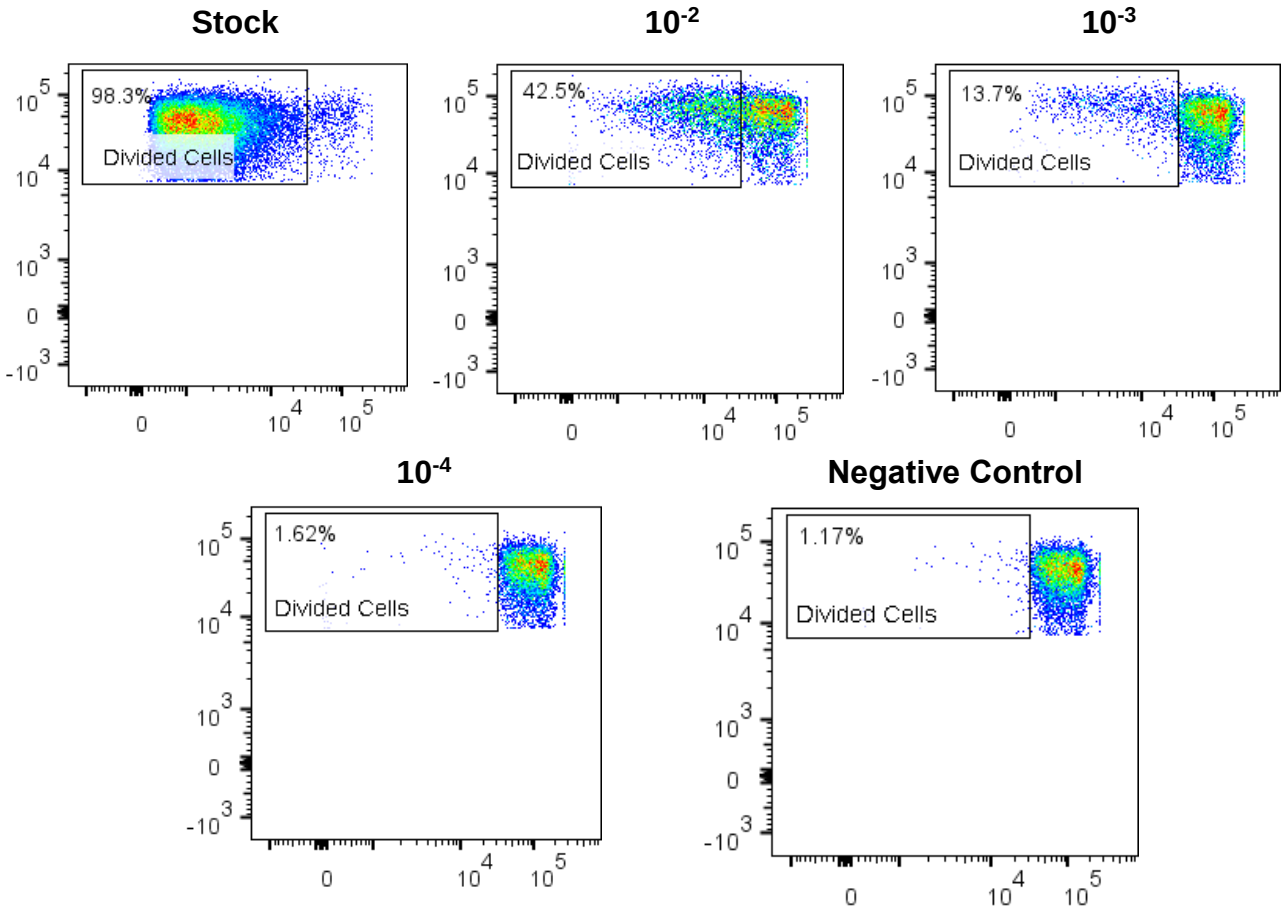

Figure 4.9 - Dilution of POL peptide *in vivo*

6- to 8-week old BALB/c mice received 3-5 x10^6 MACS-purified, CTV-labelled naïve CL4 CD8+ T cells by adoptive transfer followed by POL peptide at different concentrations (stock: 200 µM, 2-fold, 3-fold or 4-fold dilutions) or PBS only (negative control). After 3 days CD8+ T cells were extracted from spleens using MACS, stained using specific mAbs for flow cytometry and analysed using FlowJo v10.3.

Data shown is from one single experiment where n=1.

here than in previous experiments to be able to elicit an appropriate response from transferred CD8+ T cells and to account for the difference in the *in vitro* and *in vivo* experimental settings.

As shown, varying magnitudes of responses were observed across different POL concentrations. The proliferation observed in mice treated with 4-fold diluted POL was very low and very similar to that of the negative control. On the other hand, POL at stock concentration caused CD8+ T cell proliferation so strongly that cells were close to losing the CTV dye due to extensive dividing. POL at 10^{-2} (2 µM) and 10^{-3} (200 nM) dilutions were the middle ground as these concentrations had elicited sufficient CD8+ T cell proliferation without cells dividing too rapidly to lose CTV. Hence, concentrations 2 µM and 200 nM were identified to be used in subsequent experiments for comparison with POL-SAGE.

Based on these results, we assessed the division of CD8+ T cells in mice treated with SAGE-POL at 2 µM or 200 nM to identify whether the proliferative response could be improved compared to that of POL peptide alone. To do so, BALB/c mice received adoptive transfer of CTV-labelled CD8+ T cells followed by treatment with either POL peptide alone or POL-SAGE, at one of the two concentrations (2 µM or 200 nM). After 3 days, CD8+ cells were extracted using MACS and stained for flow cytometry using specific mAbs (Figure 4.10). The percentage of CTV-negative cells is representative of proliferated cells that have undergone sufficient rounds of division to lose the CTV dye. Thus, at either concentration, 2 µM or 200 nM, POL-SAGE evoked a more proliferative response than POL peptide alone as can be seen by the higher percentages of CTV-negative cells in samples treated with POL-SAGE. This shows that conjugation to the SAGE particle improved the proliferative response of CD8+ T cells compared to POL peptide alone.

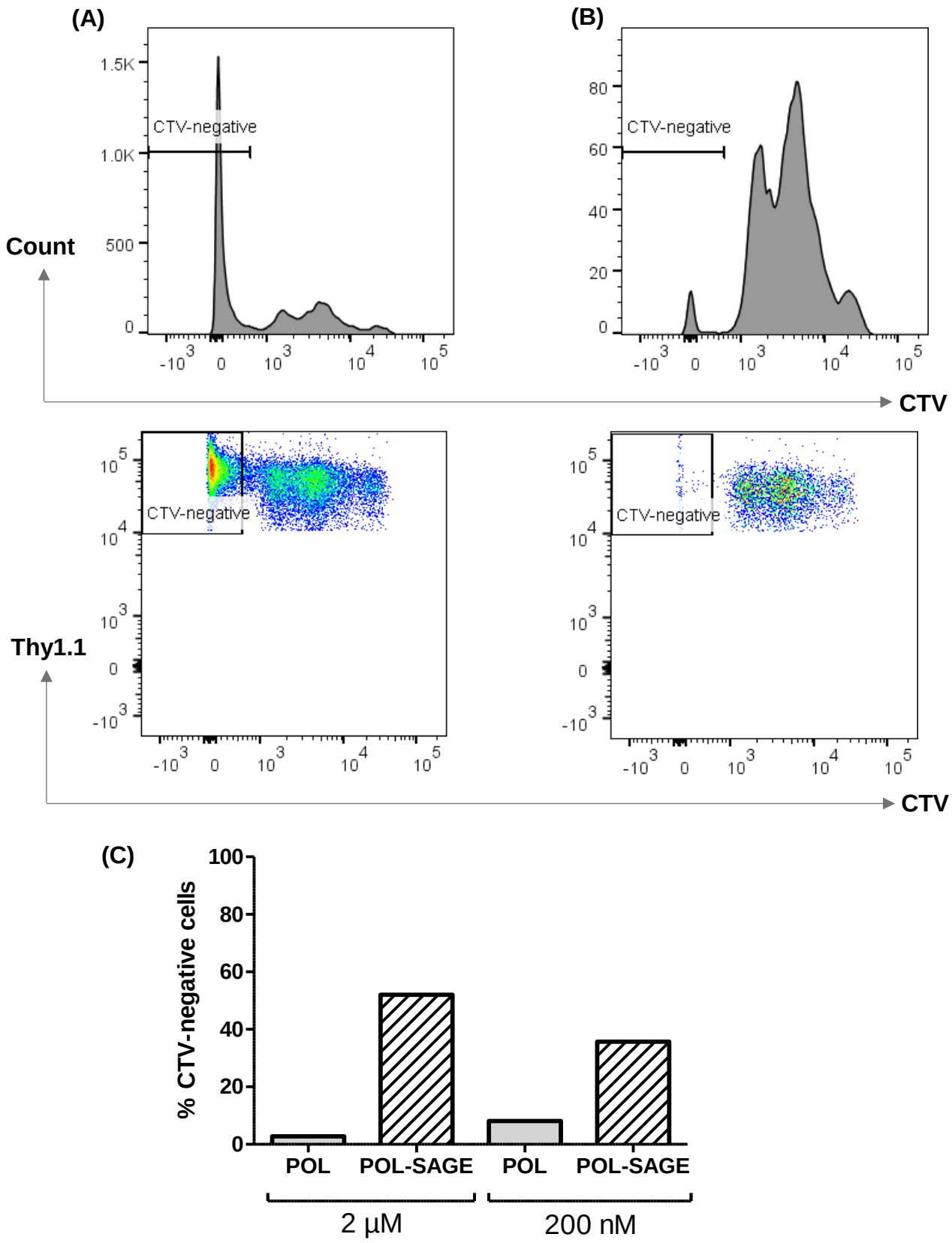

Figure 4.10 - Comparing CD8+ T cell proliferative response of POL-SAGE to POL Peptide alone

6- to 8-week old BALB/c mice received 3-5 x10^6 MACS-purified, CTV-labelled naïve CL4 CD8+ T cells by adoptive transfer followed by either POL peptide (at 2 µM or 200 nM) or POL-SAGE (at 2 µM or 200 nM). 3 days later CD8+ T cells were extracted from spleens using MACS, stained using specific mAbs for flow cytometry. (A) and (B) show flow cytometry data from mice treated with 2 µM POL-SAGE or 2 µM POL peptide only, respectively. (C) is a bar graph showing the percentages of CTV-negative cells of the different conditions.
Data shown is from one single experiment where n=1.

4.6 Discussion

The presence of high levels of TILS in tumours has been correlated with good prognoses in a large variety of cancers (81, 83). Thus, many approaches of immunotherapeutic vaccines aim to generate large numbers of anti-tumour CTLs and to strengthen their effector functions to provide anti-tumour immunity. Often, this involves targeting DCs due to their efficiency in antigen uptake, processing and presentation via MHC class I molecules to CD8+ T cells (132, 133). Approaches to DC-based therapies include the delivery of tumour antigens in combination with DC stimulant (e.g. Provenge) in prostate cancer (110) and optimisation of tumour RNA-loaded DCs *ex vivo* followed by injection to the patient to initiate antigen-presentation to anti-tumour CTL (141).

SAGEs are novel protein scaffolds formed of *de novo* coiled-coil peptides. They are composed of two hubs that assemble into a cage which can contain or have material attached to. Their components are highly interchangeable, allowing customisation of the particles for a range of needs. The versatility of SAGE particles provides potential for its uses in an anti-tumour context. Due to the hub being very flexible in what can be constructed with or enclosed within it, this presents the capacity to develop a vaccine with SAGEs conjugated to tumour-antigens to increase the number of anti-tumour CTLs by driving their proliferation. Induction of central tolerance hinders excessive autoimmunity but inadvertently also leads to the tolerization of anti-tumour CTLs. This means that the few CTLs that do escape this process also exhibit low TCR affinity. Thus it is essential for a cancer vaccine to establish high numbers of long-lived anti-tumour CTLs with high affinity TCRs to fight the tumour.

In this study we investigated the ability of SAGEs to initiate proliferation of CD8+ T cells *in vivo.* Based on the work of de Haan *et al* (2002) (136), three different length peptides, EP, POL and LEA, were initially tested for their ability to initiate CD8+ T cell proliferation *in vitro*. Due to uptake and processing of the peptides prior to displaying when using irradiated Renca cells, P815 cells were used instead. Culturing CD8+ T cells with these MHC class I-possessing cells that had been pulsed with EP, POL and LEA elicited strong CD8+ T cell proliferative responses, confirming the peptides' ability to be recognised by the TCR. A cathepsin cleavage site had been added into the LEA peptide to potentially improve peptide uptake via SAGEs by APCs. However, we found that the loop contained in POL was sufficient to allow targeting of SAGEs and elicited abundant CD8+ T cell proliferation. We found that despite some immunogenicity in other settings (e.g. when cultured with PBMCs; (116)), SAGEs did not prime CD8+ T cells in an unconjugated state. This finding ensures that proliferation observed in experiments was due to conjugation of peptides to the SAGE particle. Conjugation of POL peptide to SAGE molecules elicited higher proliferation than when using POL peptide alone *in vivo*, driving strong CD8+ T

cell responses specific to the conjugated peptide. This is an important characteristic to consider in the context of inducing effective anti-tumour CTLs. Since tumour-specific CTLs often exhibit low TCR specificity due to central tolerance, strong TCR stimulation as well as high proliferation of CTLs using peptide-conjugated SAGEs at the priming stage could improve targeted cytotoxic killing activity of CD8+ cells (75, 132). In addition to this, the observation that conjugation of peptides to SAGE drastically improved the proliferative response than when free peptide only is injected is very significant. Vaccination of free peptide alone poses the risk of peripheral tolerization due to systemic distribution where T cells become unresponsive to antigen when exposed to high doses of it repeatedly (135), thus it is important to explore means of delivery of the peptide in an effective and contained manner. Investigation whether tolerization of effector cells occurs following SAGE injection should be strongly considered, however due to the lack of adjuvant needed with peptide-conjugated SAGE injections it is possible that this issue is reduced. The results of this study highlight the great potential of peptide-conjugated SAGEs in a therapeutic setting. However, it is essential to further and repeatedly test SAGEs to be able to draw representative conclusions. Furthermore, it is important to note that therapies to increase the abundance and effector functions of CTLs should be used in conjunction with therapies targeting the immunosuppression experienced by CTLs to prevent their switching off in the TME.

To sum up, we established that conjugated SAGEs are capable of initiating sufficient proliferation of specific CD8+ T cells *in vivo* and that this response is of greater magnitude than when using peptide alone. Our findings demonstrate the potential of using SAGEs in anti-tumour immunity and whilst these initial results are very promising it is necessary to further test their ability to be able to develop and use SAGEs in a therapeutic setting.

Chapter 5 - Future Work

CTLs are activated when their TCR binds tumour-antigen presented via MHC class I by APCs in tumour-draining lymph nodes. Activated CTLs then travel to the site of the tumour to infiltrate the tumour stroma in an attempt to perform their cytotoxic functions. However, in the presence of immunosuppressive factors such as areas of high numbers of T_{reg} cells, high adenosine concentrations and anti-inflammatory factors, CTLs lose their ability to perform effector function and display increased CIR expression on their surface. This results in tumour tolerance where the tumour is not attacked and is thus able to continue to grow without interference. Tumour vaccinations aim to encourage the host's own immune system to restore its anti-tumour function by targeting a variety of immune cells. T-cell-targeted tumour vaccines provide a viable route to cancer treatment as they aim to activate anti-tumour CD8+ T cells strongly to attempt restoration of their cytotoxic ability and prevent them from becoming exhausted.

The work in this thesis was carried out in order to identify how adenosine influences CD8+ T cells towards the exhausted phenotype and how adenosine and CIR expression are related. We found no correlation between adenosine concentration and CIR expression on CD8+ T cell surface. Additionally, we found no effect of adenosine on the priming and proliferation of CD8+ T cells. Furthermore, we investigated how the anti-tumour response of CD8+ T cells could be improved and strengthened using SAGE molecules. Our data show that antigen-conjugated SAGEs were capable of initiating strong proliferative responses of CD8+ T cells *in vivo*.

5.1 Adenosine

Our experiments found no effect of the adenosine receptor agonist NECA on the proliferative ability of CD8+ T cells. Although their proliferation was unaffected, their other effector functions were not investigated. Thus, this should be investigated in the future by conducting tumour cell killing assay using CD8+ T cells that were primed in the presence of adenosine. Further, NECA not affecting CD8+ T cell proliferation could be due to the possible involvement of A3 adenosine receptors in the suppression of CD8+ T cells (43). This contribution has been debated however, thus it is important to establish whether signalling via A3 adenosine receptors can affect CD8+ T cell function. No changes were seen in the expression of CIRs when cells were activated in the presence or absence of NECA, suggesting no direct link between the two inhibitory factors in CTLs. Understanding the expression patterns of CIRs on CD8+ T cells is crucial to be able to use them as biomarkers of exhausted cells in cancer treatments. Co-expression of CIRs plays a crucial role in the limited success of checkpoint blockade therapy, as it provides resilience to the inhibitory pathways. Thus, it is important to characterise these co-expression patterns by

also assessing tumour-infiltrating lymphocytes and their CIR profiles prior to and during exhaustion. Our findings highlight the urgency of understanding the underlying mechanisms in the TME that cooperate to elicit immunosuppression to be able to develop therapies against them.

5.2 SAGEs

Our investigations found that SAGEs are capable of eliciting strong proliferative responses from specific CD8+ T cells. These initial results are very promising and open up further development of the use of SAGEs against tumours. In the context of our experiments, first and foremost it is essential to conduct more repeats *in vivo* using POL-SAGEs to increase the reliability of our findings. Furthermore, characterisation of CD8+ T cells activated using SAGEs is also important in order to assess which kind of effector cells they are. This could be done by labelling cells for activation, effector and memory markers to identify their characteristics using flow cytometry. The functionality of the primed CD8+ T cells must also be established, such as their cytotoxic ability in tumour killing assays. An ideal tumour vaccine would establish CTLs with TCRs exhibiting high affinity and avidity to tumour antigens presented to them via MHC complexes. Additionally, these cells would secrete high concentrations of cytotoxic factors (e.g. granzymes), would display molecules for T cell migration to the tumour site on their surface which would be maintains, such as integrins and CXCR3 (142-144), and would express high levels of costimulatory molecules and low levels of CIRs (132). Additionally, after proliferation of CD8+ T cells *in vivo* the numbers of DCs should be assessed to determine the longevity of the response. Strong activation of APCs such as DCs provides the potential to establish lasting anti-tumour immunity (139). Following from this, it is important to identify which APCs SAGEs target by e.g. fusing SAGEs with GFP or mCherry to help their localisation (115). Further, SAGE-induced CD8+ T cells should be examined in a tumour model to compare tumour growth in mice given adoptive transfer with or without SAGE. Or alternatively, mice should be inoculated with SAGEs prior to challenging the system with tumour cells to assess the possibility of using SAGEs in a prophylactic manner.

5.3 Therapies

Our findings showed that SAGEs can be used to initiate CD8+ T cell proliferative responses *in vivo.* This is very encouraging for the potential use of SAGEs as T-cell-inducing vaccines, either in the treatment of tumours or preventatively (132). By establishing a strong TCR signal during CTL activation, SAGEs could help reinstate the anti-tumour functions of CTLs (75, 132). It is important to consider that despite the CTL activation ability of SAGEs, tolerization is still possible. However due to the lack of adjuvant needed for potency of SAGE, this is unlikely. Furthermore, a large limitation in the development of tumour vaccines is the identification of

tumour antigens. Thus, this highlights the importance of identifying antigens consistently expressed on and specific to tumours to be able to efficiently target therapies against them.

Based on the success of combining different immunotherapeutic approaches, such as the combination of Nivolumab with chemotherapy, it is important to not see current therapies as different entities but instead consider their synergism together to achieve lasting anti-tumour immunity.

Chapter 6 - Appendix

Figure 6.1 - Proposed model of suppression of CL4 CD8+ T cells by adenosine production by tumour infiltrating T_{reg} cells

Within the TDLN, T_{reg} cells express lower levels of ectoenzymes CD39 and 5'nucleotidase. CD39 catalyses the breakdown of ATP into ADP as well as ADP into AMP. AMP is in turn broken down to adenosine by 5'nucleotidase. CL4 CD8+ T cells express adenosine receptor A2AR. However, within the tumour, high levels of CD39 and 5'nucleotidase result in elevated production and accumulation of adenosine, which binds to A2AR receptors on CL4 CD8+ T cells and causes suppression of anti-tumour function.

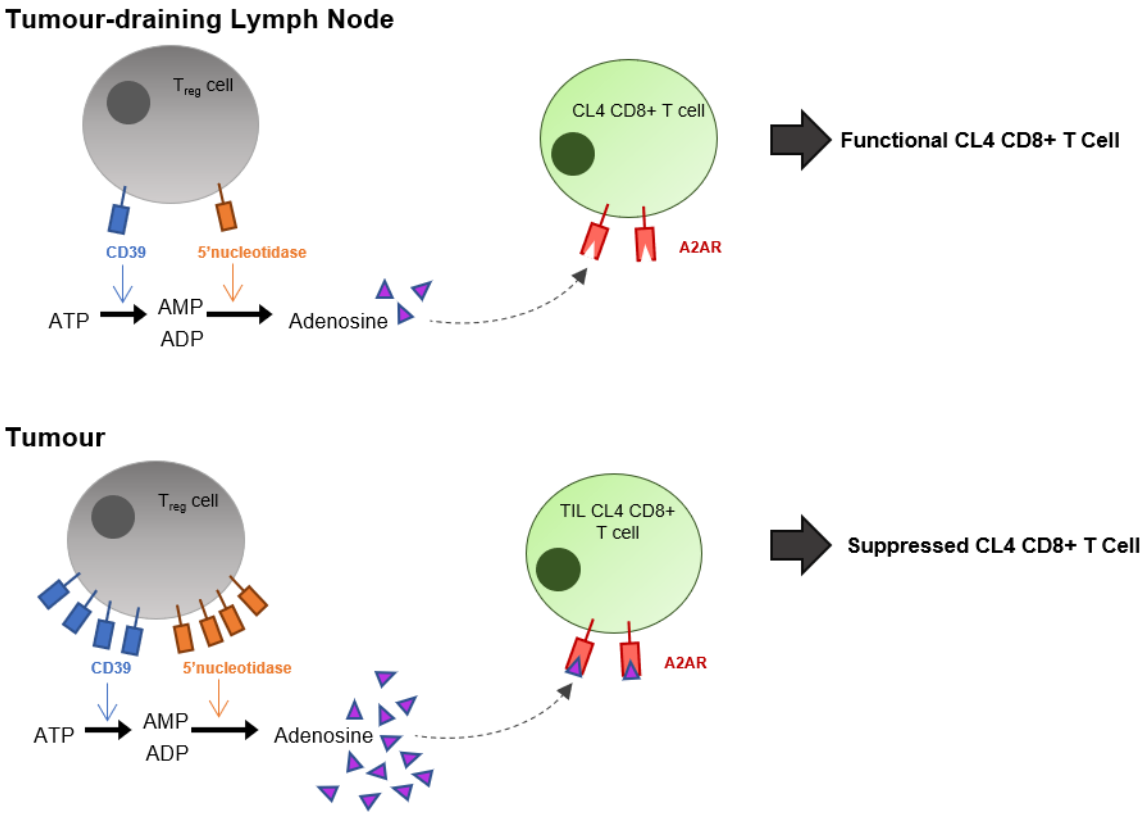

Figure 6.1 - Proposed model of suppression of CL4 CD8+ T cells by adenosine production by tumour-infiltrating Treg cells

Figure 6.2 - Gating strategy for CL4 mouse typing

Blood samples were collected from the tail veins of CL4 mice. Following erythrocyte lysis, blood was stained using fluorescently-labelled mAbs specific for CD8α and Vβ8.1 to analyse CL4 TCR gene expression. CL4 mice (A) have higher percentages of $CD8\alpha^{+}V\beta8.1^{+}$ cells (Q2) than CL4-negative mice (B).

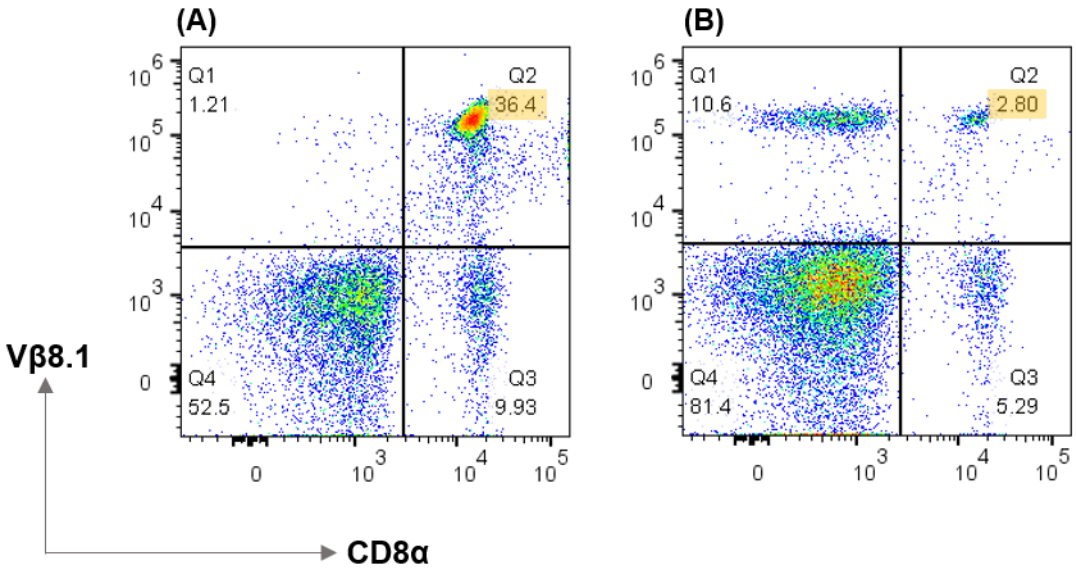

Figure 6.2 - Gating Strategy for CL4 mouse typing

Figure 6.3 - Enrichment Purity of MACS

Positive enrichment of CL4 CD8+ T cells was performed in experiments as indicated. After isolation of cells from spleens, CD8+ T cells were bound to anti-CD8-labelled magnetic microbeads and purified using magnetic-activated sorting. This involves the application of the cells through a magnetic column in a magnetic field, separating cells of interest out of suspension by positive selection which are subsequently flushed out. Enrichment Purity of this method was routinely ~95% (Q2).

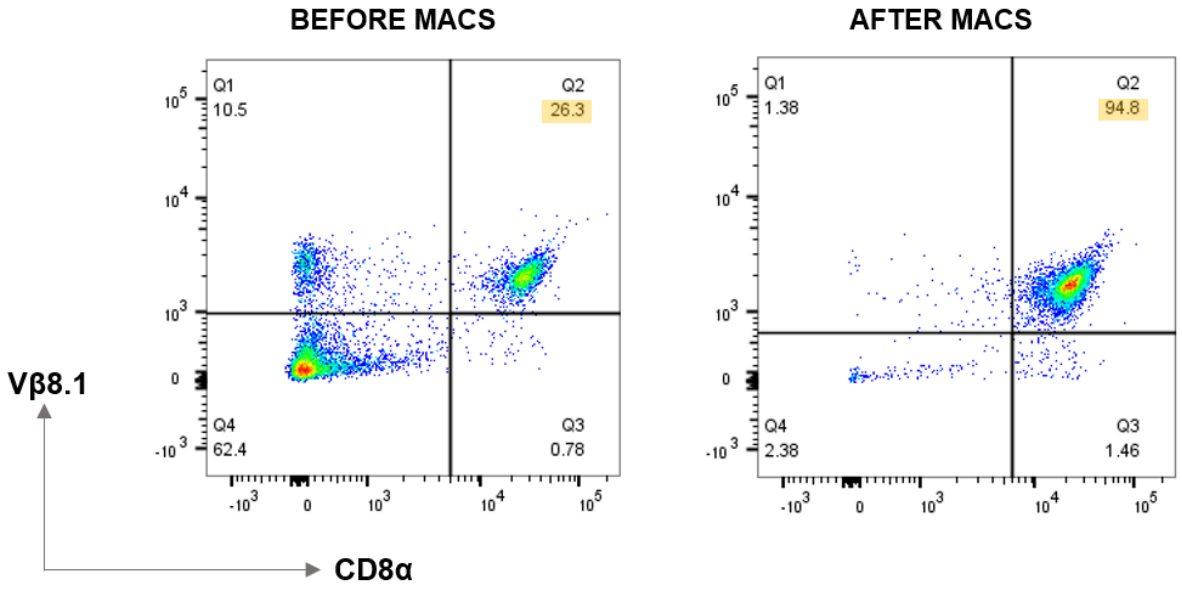

Figure 6.3 - Enrichment Purity of MACS

Table 6.1 - Anti-mouse antibodies used in FACS

Antibody	Fluorochrome	Dilution	Source	Clone
CD8α	FITC	1 in 100	Biolegend	53-6.7
CD8β	PE-Cy7	1 in 200	Biolegend	53-5.8
LAG3	PE-Cy7	1 in 200	eBiosciences	C9B7W
PD1	BV785	1 in 200	Biolegend	29F.1A12
THY1.1	PerCP Cy5.5	1 in 100	Biolegend	OX-7
TIGIT	APC	1 in 100	Biolegend	IG9
TIM3	BV605	1 in 100	Biolegend	RMT3-23
TIM3	PE	1 in 100	Biolegend	B8.2C12
Vβ8.1	FITC	1 in 200	eBiosciences	KJ16-133

Figure 6.4 - Gating CD8+ T cell Proliferation

$1x10^6$ MACS-purified CL4 CD8+ T cells were labelled with CTV and subsequently activated using either anti-CD3 and anti-CD28 antibodies or pulsed splenocyte reaction (PSR). Cells were harvested after 72 hours of culture and prepared for flow cytometry analysis using fluorochrome-conjugated anti-CD8 mAbs.

CTV binds free amines on and within the cell so that as cells undergo cell division, the dye is split between the daughter cells. Thus, undivided cells are more fluorescent than cells having divided, with fluorescence decreasing the more cells have proliferated. This generates several populations of cells of different degrees of fluorescence depending on the number of divisions they have undergone.

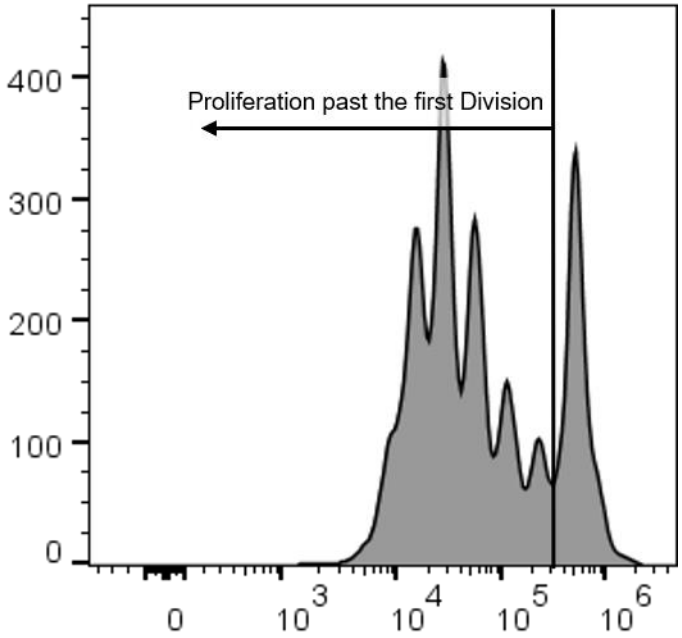

Figure 6.4 - Gating CD8+ T cell Proliferation

Chapter 7 - Bibliography

1. Yoon H, Kim TS, Braciale TJ. The Cell Cycle Time of CD8+ T Cells Responding In Vivo Is Controlled by the Type of Antigenic Stimulus. PLOS ONE. 2010;5(11):e15423.

2. Sabins NC, Chornoguz O, Leander K, Kaplan F, Carter R, Kinder M, et al. TIM-3 Engagement Promotes Effector Memory T Cell Differentiation of Human Antigen-Specific CD8 T Cells by Activating mTORC1. 2017;199(12):4091-102.

3. Virgin HW, Wherry EJ, Ahmed R. Redefining Chronic Viral Infection. Cell. 2009;138(1):30-50.

4. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol. 2004;22:745-63.

5. Jung S, Unutmaz D, Wong P, Sano G-I, De los Santos K, Sparwasser T, et al. In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens. Immunity. 2002;17(2):211-20.

6. Joffre OP, Segura E, Savina A, Amigorena S. Cross-presentation by dendritic cells. Nature Reviews Immunology. 2012;12:557.

7. Lowin B, Hahne M, Mattmann C, Tschopp J. Cytolytic T-cell cytotoxicity is mediated through perforin and Fas lytic pathways. Nature. 1994;370:650.

8. Barry M, Bleackley RC. Cytotoxic T lymphocytes: all roads lead to death. Nature Reviews Immunology. 2002;2:401.

9. Flavell RA, Sanjabi S, Wrzesinski SH, Licona-Limon P. The polarization of immune cells in the tumour environment by TGFbeta. Nature reviews Immunology. 2010;10(8):554-67.

10. Cancer Research UK - Cancer Statistics for the UK (Accessed October 2018) [Available from: https://www.cancerresearchuk.org/health-professional/cancer-statistics-for-the-uk#heading-Three.

11. Yoo HJ, Ahn SH, Kim SB, Kim WK, Han OS. Efficacy of progressive muscle relaxation training and guided imagery in reducing chemotherapy side effects in patients with breast cancer and in improving their quality of life. Supportive care in cancer : official journal of the Multinational Association of Supportive Care in Cancer. 2005;13(10):826-33.

12. Demetri GD, von Mehren M, Blanke CD, Van den Abbeele AD, Eisenberg B, Roberts PJ, et al. Efficacy and Safety of Imatinib Mesylate in Advanced Gastrointestinal Stromal Tumors. 2002;347(7):472-80.

13. Chen Z, Wu Z, Ning W. Advances in Molecular Mechanisms and Treatment of Radiation-Induced Pulmonary Fibrosis. Translational Oncology. 2019;12(1):162-9.

14. Manapov F, Käsmann L, Roengvoraphoj O, Dantes M, Schmidt-Hegemann N-S, Belka C, et al. Prophylactic cranial irradiation in small-cell lung cancer: update on patient selection, efficacy and outcomes. Lung Cancer (Auckland, NZ). 2018;9:49-55.

15. Farkona S, Diamandis EP, Blasutig IM. Cancer immunotherapy: the beginning of the end of cancer? BMC Medicine. 2016;14:73.

16. Fritsch EF, Rajasagi M, Ott PA, Brusic V, Hacohen N, Wu CJ. HLA-binding properties of tumor neoepitopes in humans. Cancer immunology research. 2014;2(6):522-9.

17. Wilson EB, El-Jawhari JJ, Neilson AL, Hall GD, Melcher AA, Meade JL, et al. Human Tumour Immune Evasion via TGF-β Blocks NK Cell Activation but Not Survival Allowing Therapeutic Restoration of Anti-Tumour Activity. PLOS ONE. 2011;6(9):e22842.

18. Waldhauer I, Steinle A. NK cells and cancer immunosurveillance. Oncogene. 2008;27:5932.

19. Raulet DH, Guerra N. Oncogenic stress sensed by the immune system: role of natural killer cell receptors. Nature Reviews Immunology. 2009;9:568.

20. Dunn GP, Old LJ, Schreiber RD. The Immunobiology of Cancer Immunosurveillance and Immunoediting. Immunity. 2004;21(2):137-48.

21. Dunn GP, Old LJ, Schreiber RD. The Three Es of Cancer Immunoediting. Annual Review of Immunology. 2004;22(1):329-60.

22. Schreiber RD, Old LJ, Smyth MJ. Cancer Immunoediting: Integrating Immunity's Roles in Cancer Suppression and Promotion. 2011;331(6024):1565-70.

23. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nature Immunology. 2002;3:991.

24. Ahmadzadeh M, Johnson LA, Heemskerk B, Wunderlich JR, Dudley ME, White DE, et al. Tumor antigen–specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. 2009;114(8):1537-44.

25. Uribe D, Torres Á, Rocha JD, Niechi I, Oyarzún C, Sobrevia L, et al. Multidrug resistance in glioblastoma stem-like cells: Role of the hypoxic microenvironment and adenosine signaling. Molecular aspects of medicine. 2017;55:140-51.

26. Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nature Medicine. 2004;10:942.

27. Shimizu J, Yamazaki S, Sakaguchi S. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. Journal of immunology (Baltimore, Md : 1950). 1999;163(10):5211-8.

28. Steitz J, Bruck J, Lenz J, Knop J, Tuting T. Depletion of CD25(+) CD4(+) T cells and treatment with tyrosinase-related protein 2-transduced dendritic cells enhance the interferon alpha-induced, CD8(+) T-cell-dependent immune defense of B16 melanoma. Cancer research. 2001;61(24):8643-6.

29. Allavena P, Mantovani A. Immunology in the clinic review series; focus on cancer: tumour-associated macrophages: undisputed stars of the inflammatory tumour microenvironment. 2012;167(2):195-205.

30. Singer M, Wang C, Cong L, Marjanovic ND, Kowalczyk MS, Zhang H, et al. A Distinct Gene Module for Dysfunction Uncoupled from Activation in Tumor-Infiltrating T Cells. Cell. 2016;166(6):1500-11.e9.

31. Schwartz RH. T cell anergy. Annu Rev Immunol. 2003;21:305-34.

32. Li J, Shayan G, Avery L, Jie H-B, Gildener-Leapman N, Schmitt N, et al. Tumor-infiltrating Tim-3+ T cells proliferate avidly except when PD-1 is co-expressed: Evidence for intracellular cross talk. OncoImmunology. 2016;5(10):e1200778.

33. Thommen DS, Schreiner J, Muller P, Herzig P, Roller A, Belousov A, et al. Progression of Lung Cancer Is Associated with Increased Dysfunction of T Cells Defined by Coexpression of Multiple Inhibitory Receptors. Cancer immunology research. 2015;3(12):1344-55.

34. Mediavilla-Varela M, Castro J, Chiappori A, Noyes D, Hernandez DC, Allard B, et al. A Novel Antagonist of the Immune Checkpoint Protein Adenosine A2a Receptor Restores Tumor-Infiltrating Lymphocyte Activity in the Context of the Tumor Microenvironment. Neoplasia (New York, NY). 2017;19(7):530-6.

35. Fredholm BB. Adenosine receptors as drug targets. Experimental cell research. 2010;316(8):1284-8.

36. Bavaresco L, Bernardi A, Braganhol E, Cappellari AR, Rockenbach L, Farias PF, et al. The role of ecto-5′-nucleotidase/CD73 in glioma cell line proliferation. Molecular and Cellular Biochemistry. 2008;319(1):61-8.

37. Blay J, White TD, Hoskin DW. The extracellular fluid of solid carcinomas contains immunosuppressive concentrations of adenosine. Cancer research. 1997;57(13):2602-5.

38. Jin D, Fan J, Wang L, Thompson LF, Liu A, Daniel BJ, et al. CD73 on Tumor Cells Impairs Antitumor T-Cell Responses: A Novel Mechanism of Tumor-Induced Immune Suppression. 2010;70(6):2245-55.

39. Zhang B. CD73: A Novel Target for Cancer Immunotherapy. 2010;70(16):6407-11.

40. Boison D. Adenosine as a neuromodulator in neurological diseases. Current Opinion in Pharmacology. 2008;8(1):2-7.

41. Young A, Mittal D, Stannard K, Yong M, Teng MWL, Allard B, et al. Co-blockade of immune checkpoints and adenosine A2A receptor suppresses metastasis. OncoImmunology. 2014;3(10):e958952.

42. Jacobson KA, Gao ZG. Adenosine receptors as therapeutic targets. Nature reviews Drug discovery. 2006;5(3):247-64.

43. Hoskin DW, Butler JJ, Drapeau D, Haeryfar SMM, Blay J. Adenosine acts through an A3 receptor to prevent the induction of murine anti-CD3-activated killer T cells. International journal of cancer. 2002;99(3):386-95.

44. Bowser JL, Phan LH, Eltzschig HK. The Hypoxia–Adenosine Link during Intestinal Inflammation. 2018;200(3):897-907.

45. Fredholm BB, Abbracchio MP, Burnstock G, Daly JW, Harden TK, Jacobson KA, et al. Nomenclature and classification of purinoceptors. Pharmacological reviews. 1994;46(2):143-56.

46. Allard B, Pommey S, Smyth MJ, Stagg J. Targeting CD73 enhances the antitumor activity of anti-PD-1 and anti-CTLA-4 mAbs. Clinical cancer research : an official journal of the American Association for Cancer Research. 2013;19(20):5626-35.

47. Takahashi RN, Pamplona FA, Prediger RD. Adenosine receptor antagonists for cognitive dysfunction: a review of animal studies. Frontiers in bioscience : a journal and virtual library. 2008;13:2614-32.

48. Vijayan D, Young A, Teng MWL, Smyth MJ. Targeting immunosuppressive adenosine in cancer. Nature Reviews Cancer. 2017;17:709.

49. Ohta A, Gorelik E, Prasad SJ, Ronchese F, Lukashev D, Wong MK, et al. A2A adenosine receptor protects tumors from antitumor T cells. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(35):13132-7.

50. Minkowski MD, Castellazzi M, Buttin G. Lack of adenosine deaminase activity in cultured murine cytotoxic T lymphocytes. Journal of immunology (Baltimore, Md : 1950). 1984;133(1):52-8.

51. Dong RP, Kameoka J, Hegen M, Tanaka T, Xu Y, Schlossman SF, et al. Characterization of adenosine deaminase binding to human CD26 on T cells and its biologic role in immune response. Journal of immunology (Baltimore, Md : 1950). 1996;156(4):1349-55.

52. Allard D, Turcotte M, Stagg J. Targeting A2 adenosine receptors in cancer. Immunology and cell biology. 2017;95(4):333-9.

53. Tassi E, Grazia G, Vegetti C, Bersani I, Bertolini G, Molla A, et al. Early Effector T Lymphocytes Coexpress Multiple Inhibitory Receptors in Primary Non-Small Cell Lung Cancer. Cancer research. 2017;77(4):851-61.

54. McFarland HF, Martin R. Multiple sclerosis: a complicated picture of autoimmunity. Nature Immunology. 2007;8:913.

55. Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. Annu Rev Immunol. 2005;23:515-48.

56. Zhang J-Y, Zhang Z, Wang X, Fu J-L, Yao J, Jiao Y, et al. PD-1 up-regulation is correlated with HIV-specific memory CD8⁺ T-cell exhaustion in typical progressors but not in long-term nonprogressors. 2007;109(11):4671-8.

57. Parry RV, Chemnitz JM, Frauwirth KA, Lanfranco AR, Braunstein I, Kobayashi SV, et al. CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. Molecular and cellular biology. 2005;25(21):9543-53.

58. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. Annu Rev Immunol. 2008;26:677-704.

59. Kurtulus S, Sakuishi K, Ngiow S-F, Joller N, Tan DJ, Teng MWL, et al. TIGIT predominantly regulates the immune response via regulatory T cells. The Journal of Clinical Investigation. 2015;125(11):4053-62.

60. Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ. Programmed Death-1 Ligand 1 Interacts Specifically with the B7-1 Costimulatory Molecule to Inhibit T Cell Responses. Immunity. 2007;27(1):111-22.

61. Mahoney KM, Rennert PD, Freeman GJ. Combination cancer immunotherapy and new immunomodulatory targets. Nature reviews Drug discovery. 2015;14(8):561-84.

62. Nurieva R, Thomas S, Nguyen T, Martin-Orozco N, Wang Y, Kaja MK, et al. T-cell tolerance or function is determined by combinatorial costimulatory signals. The EMBO Journal. 2006;25(11):2623-33.

63. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. The New England journal of medicine. 2012;366(26):2443-54.

64. Sakuishi K, Apetoh L, Sullivan JM, Blazar BR, Kuchroo VK, Anderson AC. Targeting Tim-3 and PD-1 pathways to reverse T cell exhaustion and restore anti-tumor immunity. The Journal of experimental medicine. 2010;207(10):2187-94.

65. Yang Z-Z, Grote DM, Ziesmer SC, Niki T, Hirashima M, Novak AJ, et al. IL-12 upregulates TIM-3 expression and induces T cell exhaustion in patients with follicular B cell non-Hodgkin lymphoma. The Journal of Clinical Investigation. 2012;122(4):1271-82.

66. Khan H, Gucalp R, Shapira I. Evolving Concepts: Immunity in Oncology from Targets to Treatments. Journal of Oncology. 2015;2015:847383.

67. Odorizzi PM, Pauken KE, Paley MA, Sharpe A, Wherry EJ. Genetic absence of PD-1 promotes accumulation of terminally differentiated exhausted CD8(+) T cells. The Journal of experimental medicine. 2015;212(7):1125-37.

68. Lipson EJ, Forde PM, Hammers HJ, Emens LA, Taube JM, Topalian SL. Antagonists of PD-1 and PD-L1 in Cancer Treatment. Seminars in oncology. 2015;42(4):587-600.

69. Sakuishi K, Ngiow SF, Sullivan JM, Teng MW, Kuchroo VK, Smyth MJ, et al. TIM3(+)FOXP3(+) regulatory T cells are tissue-specific promoters of T-cell dysfunction in cancer. Oncoimmunology. 2013;2(4):e23849.

70. Hemon P, Jean-Louis F, Ramgolam K, Brignone C, Viguier M, Bachelez H, et al. MHC Class II Engagement by Its Ligand LAG-3 (CD223) Contributes to Melanoma Resistance to Apoptosis. 2011.

71. Darlington PJ, Baroja ML, Chau TA, Siu E, Ling V, Carreno BM, et al. Surface cytotoxic T lymphocyte-associated antigen 4 partitions within lipid rafts and relocates to the immunological synapse under conditions of inhibition of T cell activation. The Journal of experimental medicine. 2002;195(10):1337-47.

72. Chauvin J-M, Pagliano O, Fourcade J, Sun Z, Wang H, Sander C, et al. TIGIT and PD-1 impair tumor antigen–specific CD8+ T cells in melanoma patients. The Journal of Clinical Investigation. 2015;125(5):2046-58.

73. Bottino C, Castriconi R, Pende D, Rivera P, Nanni M, Carnemolla B, et al. Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. The Journal of experimental medicine. 2003;198(4):557-67.

74. Leitner J, Rieger A, Pickl WF, Zlabinger G, Grabmeier-Pfistershammer K, Steinberger P. TIM-3 does not act as a receptor for galectin-9. PLoS pathogens. 2013;9(3):e1003253.

75. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity. 2013;39(1):1-10.

76. Mellman I, Coukos G, Dranoff G. Cancer immunotherapy comes of age. Nature. 2011;480(7378):480-9.

77. Rosenberg SA, Yang JC, Sherry RM, Kammula US, Hughes MS, Phan GQ, et al. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. Clinical cancer research : an official journal of the American Association for Cancer Research. 2011;17(13):4550-7.

78. Kalos M, June Carl H. Adoptive T Cell Transfer for Cancer Immunotherapy in the Era of Synthetic Biology. Immunity. 2013;39(1):49-60.

79. Gross G, Waks T, Eshhar Z. Expression of immunoglobulin-T-cell receptor chimeric molecules as functional receptors with antibody-type specificity. Proceedings of the National Academy of Sciences of the United States of America. 1989;86(24):10024-8.

80. Hinrichs CS, Rosenberg SA. Exploiting the curative potential of adoptive T-cell therapy for cancer. Immunol Rev. 2014;257(1):56-71.

81. Chen Z, Chen X, Zhou E, Chen G, Qian K, Wu X, et al. Intratumoral $CD8^+$ cytotoxic lymphocyte is a favorable prognostic marker in node-negative breast cancer. PloS one. 2014;9(4):e95475-e.

82. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science (New York, NY). 2006;313(5795):1960-4.

83. Hiraoka K, Miyamoto M, Cho Y, Suzuoki M, Oshikiri T, Nakakubo Y, et al. Concurrent infiltration by CD8+ T cells and CD4+ T cells is a favourable prognostic factor in non-small-cell lung carcinoma. Br J Cancer. 2006;94(2):275-80.

84. Ashida A, Boku N, Aoyagi K, Sato H, Tsubosa Y, Minashi K, et al. Expression profiling of esophageal squamous cell carcinoma patients treated with definitive chemoradiotherapy: clinical implications. International journal of oncology. 2006;28(6):1345-52.

85. Nakano O, Sato M, Naito Y, Suzuki K, Orikasa S, Aizawa M, et al. Proliferative activity of intratumoral CD8(+) T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. Cancer research. 2001;61(13):5132-6.

86. Sabado RL, Bhardwaj N. Dendritic cell immunotherapy. 2013;1284(1):31-45.

87. Draube A, Klein-González N, Mattheus S, Brillant C, Hellmich M, Engert A, et al. Dendritic Cell Based Tumor Vaccination in Prostate and Renal Cell Cancer: A Systematic Review and Meta-Analysis. PLOS ONE. 2011;6(4):e18801.

88. Stagg J, Divisekera U, McLaughlin N, Sharkey J, Pommey S, Denoyer D, et al. Anti-CD73 antibody therapy inhibits breast tumor growth and metastasis. Proceedings of the National Academy of Sciences of the United States of America. 2010;107(4):1547-52.

89. Beavis PA, Divisekera U, Paget C, Chow MT, John LB, Devaud C, et al. Blockade of A2A receptors potently suppresses the metastasis of CD73+ tumors. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(36):14711-6.

90. Schwarzschild MA, Agnati L, Fuxe K, Chen J-F, Morelli M. Targeting adenosine A2A receptors in Parkinson's disease. Trends in Neurosciences. 2006;29(11):647-54.

91. Loi S, Pommey S, Haibe-Kains B, Beavis PA, Darcy PK, Smyth MJ, et al. CD73 promotes anthracycline resistance and poor prognosis in triple negative breast cancer. Proceedings of the National Academy of Sciences of the United States of America. 2013;110(27):11091-6.

92. Antonioli L, Yegutkin GG, Pacher P, Blandizzi C, Haskó G. Anti-CD73 in cancer immunotherapy: awakening new opportunities. Trends in cancer. 2016;2(2):95-109.

93. Blackburn SD, Shin H, Haining WN, Zou T, Workman CJ, Polley A, et al. Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection. Nat Immunol. 2009;10(1):29-37.

94. Fourcade J, Sun Z, Benallaoua M, Guillaume P, Luescher IF, Sander C, et al. Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. The Journal of experimental medicine. 2010;207(10):2175-86.

95. Fourcade J, Sun Z, Pagliano O, Guillaume P, Luescher IF, Sander C, et al. CD8(+) T cells specific for tumor antigens can be rendered dysfunctional by the tumor microenvironment through upregulation of the inhibitory receptors BTLA and PD-1. Cancer research. 2012;72(4):887-96.

96. Fourcade J, Kudela P, Sun Z, Shen H, Land SR, Lenzner D, et al. PD-1 is a regulator of NY-ESO-1-specific CD8+ T cell expansion in melanoma patients. Journal of immunology (Baltimore, Md : 1950). 2009;182(9):5240-9.

97. Topalian SL, Sznol M, McDermott DF, Kluger HM, Carvajal RD, Sharfman WH, et al. Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2014;32(10):1020-30.

98. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, et al. Nivolumab plus Ipilimumab in Advanced Melanoma. 2013;369(2):122-33.

99. Reck M, Rodríguez-Abreu D, Robinson AG, Hui R, Csőszi T, Fülöp A, et al. Pembrolizumab versus Chemotherapy for PD-L1–Positive Non–Small-Cell Lung Cancer. 2016;375(19):1823-33.

100. Sharma P, Allison JP. Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential. Cell. 2015;161(2):205-14.

101. Carbone DP, Reck M, Paz-Ares L, Creelan B, Horn L, Steins M, et al. First-Line Nivolumab in Stage IV or Recurrent Non–Small-Cell Lung Cancer. 2017;376(25):2415-26.

102. Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R, et al. Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. The New England journal of medicine. 2013;369(2):134-44.

103. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. The New England journal of medicine. 2010;363(8):711-23.

104. Hellmann MD, Rizvi NA, Goldman JW, Gettinger SN, Borghaei H, Brahmer JR, et al. Nivolumab plus ipilimumab as first-line treatment for advanced non-small-cell lung cancer (CheckMate 012): results of an open-label, phase 1, multicohort study. The Lancet Oncology. 2017;18(1):31-41.

105. Zhang B, Wu Q, Zhou YL, Guo X, Ge J, Fu J. Immune-related adverse events from combination immunotherapy in cancer patients: A comprehensive meta-analysis of randomized controlled trials. International immunopharmacology. 2018;63:292-8.

106. Preusser M, Lim M, Hafler DA, Reardon DA, Sampson JH. Prospects of immune checkpoint modulators in the treatment of glioblastoma. Nature Reviews Neurology. 2015;11:504.

107. Sharma P, Hu-Lieskovan S, Wargo JA, Ribas A. Primary, Adaptive, and Acquired Resistance to Cancer Immunotherapy. Cell. 2017;168(4):707-23.

108. Jenkins RW, Barbie DA, Flaherty KT. Mechanisms of resistance to immune checkpoint inhibitors. British Journal Of Cancer. 2018;118:9.

109. Topalian SL, Weiner GJ, Pardoll DM. Cancer immunotherapy comes of age. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2011;29(36):4828-36.

110. Cheever MA, Higano CS. PROVENGE (Sipuleucel-T) in prostate cancer: the first FDA-approved therapeutic cancer vaccine. Clinical cancer research : an official journal of the American Association for Cancer Research. 2011;17(11):3520-6.

111. Le DT, Pardoll DM, Jaffee EM. Cellular vaccine approaches. Cancer journal (Sudbury, Mass). 2010;16(4):304-10.

112. Dranoff G. GM-CSF-based cancer vaccines. 2002;188(1):147-54.

113. Thomas AM, Santarsiero LM, Lutz ER, Armstrong TD, Chen Y-C, Huang L-Q, et al. Mesothelin-specific CD8⁺ T Cell Responses Provide Evidence of In Vivo Cross-Priming by Antigen-Presenting Cells in Vaccinated Pancreatic Cancer Patients. 2004;200(3):297-306.

114. Kreiter S, Vormehr M, van de Roemer N, Diken M, Löwer M, Diekmann J, et al. Mutant MHC class II epitopes drive therapeutic immune responses to cancer. Nature. 2015;520:692.

115. Ross JF, Bridges A, Fletcher JM, Shoemark D, Alibhai D, Bray HEV, et al. Decorating Self-Assembled Peptide Cages with Proteins. ACS nano. 2017;11(8):7901-14.

116. Morris C, Glennie, S.J., Lam, H.S., Baum, H.E., Kandage, D., Williams, N.,A., Morgan, D.J., Woolfson, D.N., Davidson, A.D. vSAGE: a vaccine platform comprising self-assembled peptide cages and immunogenic peptides. Advanced Functional Materials. 2018;(under review):(under review).

117. Fletcher JM, Harniman RL, Barnes FR, Boyle AL, Collins A, Mantell J, et al. Self-assembling cages from coiled-coil peptide modules. Science (New York, NY). 2013;340(6132):595-9.

118. Morgan DJ, Liblau R, Scott B, Fleck S, McDevitt HO, Sarvetnick N, et al. CD8(+) T cell-mediated spontaneous diabetes in neonatal mice. Journal of immunology (Baltimore, Md : 1950). 1996;157(3):978-83.

119. Jenkinson SR, Williams NA, Morgan DJ. The Role of Intercellular Adhesion Molecule-1/LFA-1 Interactions in the Generation of Tumor-Specific CD8⁺ T Cell Responses. 2005;174(6):3401-7.

120. Murphy GP, Hrushesky WJ. A Murine Renal Cell Carcinoma. JNCI: Journal of the National Cancer Institute. 1973;50(4):1013-25.

121. Janicki CN, Jenkinson SR, Williams NA, Morgan DJ. Loss of CTL function among high-avidity tumor-specific CD8+ T cells following tumor infiltration. Cancer research. 2008;68(8):2993-3000.

122. Sharpe AH. Mechanisms of costimulation. Immunological Reviews. 2009;229(1):5-11.

123. Demeure CE, Wolfers J, Martin-Garcia N, Gaulard P, Triebel F. T Lymphocytes infiltrating various tumour types express the MHC class II ligand lymphocyte activation gene-3 (LAG-3): role of LAG-3/MHC class II interactions in cell–cell contacts. European Journal of Cancer. 2001;37(13):1709-18.

124. Granier C, De Guillebon E, Blanc C, Roussel H, Badoual C, Colin E, et al. Mechanisms of action and rationale for the use of checkpoint inhibitors in cancer. ESMO open. 2017;2(2):e000213-e.

125. Woo SR, Turnis ME, Goldberg MV, Bankoti J, Selby M, Nirschl CJ, et al. Immune inhibitory molecules LAG-3 and PD-1 synergistically regulate T-cell function to promote tumoral immune escape. Cancer research. 2012;72(4):917-27.

126. Mujoomdar M, Hoskin D, Blay J. Adenosine stimulation of the proliferation of colorectal carcinoma cell lines. Roles of cell density and adenosine metabolism. Biochemical pharmacology. 2003;66(9):1737-47.

127. Mittal D, Young A, Stannard K, Yong M, Teng MWL, Allard B, et al. Antimetastatic Effects of Blocking PD-1 and the Adenosine A2A Receptor. 2014;74(14):3652-8.

128. Koshiba M, Kojima H, Huang S, Apasov S, Sitkovsky MV. Memory of extracellular adenosine A2A purinergic receptor-mediated signaling in murine T cells. The Journal of biological chemistry. 1997;272(41):25881-9.

129. Huang S, Apasov S, Koshiba M, Sitkovsky M. Role of A2a extracellular adenosine receptor-mediated signaling in adenosine-mediated inhibition of T-cell activation and expansion. Blood. 1997;90(4):1600-10.

130. Steinman RM, Hawiger D, Nussenzweig MC. Tolerogenic dendritic cells. Annu Rev Immunol. 2003;21:685-711.

131. Palucka K, Banchereau J. Cancer immunotherapy via dendritic cells. Nature Reviews Cancer. 2012;12:265.

132. Palucka K, Banchereau J. Dendritic-Cell-Based Therapeutic Cancer Vaccines. Immunity. 2013;39(1):38-48.

133. Steinman RM, Dhodapkar M. Active immunization against cancer with dendritic cells: the near future. International journal of cancer. 2001;94(4):459-73.

134. Nestle FO, Banchereau J, Hart D. Dendritic cells: On the move from bench to bedside. Nat Med. 2001;7(7):761-5.

135. Toes RE, van der Voort EI, Schoenberger SP, Drijfhout JW, van Bloois L, Storm G, et al. Enhancement of tumor outgrowth through CTL tolerization after peptide vaccination is avoided by peptide presentation on dendritic cells. Journal of immunology (Baltimore, Md : 1950). 1998;160(9):4449-56.

136. De Haan L, Hearn AR, Rivett AJ, Hirst TR. Enhanced delivery of exogenous peptides into the class I antigen processing and presentation pathway. Infection and immunity. 2002;70(6):3249-58.

137. Parasar P, Wilhelm A, Rutigliano HM, Thomas AJ, Teng L, Shi B, et al. Expression of bovine non-classical major histocompatibility complex class I proteins in mouse P815 and human K562 cells. Research in Veterinary Science. 2016;107:161-70.

138. Zacharias M, Springer S. Conformational flexibility of the MHC class I alpha1-alpha2 domain in peptide bound and free states: a molecular dynamics simulation study. Biophysical journal. 2004;87(4):2203-14.

139. Zwaveling S, Ferreira Mota SC, Nouta J, Johnson M, Lipford GB, Offringa R, et al. Established human papillomavirus type 16-expressing tumors are effectively eradicated following vaccination with long peptides. Journal of immunology (Baltimore, Md : 1950). 2002;169(1):350-8.

140. Ekeruche-Makinde J, Miles JJ, van den Berg HA, Skowera A, Cole DK, Dolton G, et al. Peptide length determines the outcome of TCR/peptide-MHCI engagement. Blood. 2013;121(7):1112-23.

141. Amin A, Dudek AZ, Logan TF, Lance RS, Holzbeierlein JM, Knox JJ, et al. Survival with AGS-003, an autologous dendritic cell–based immunotherapy, in combination with sunitinib in unfavorable risk patients with advanced renal cell carcinoma (RCC): Phase 2 study results. Journal for ImmunoTherapy of Cancer. 2015;3(1):14.

142. Appay V, Douek DC, Price DA. CD8+ T cell efficacy in vaccination and disease. Nature Medicine. 2008;14:623.

143. Mullins IM, Slingluff CL, Lee JK, Garbee CF, Shu J, Anderson SG, et al. CXC Chemokine Receptor 3 Expression by Activated CD8⁺ T cells Is Associated with Survival in Melanoma Patients with Stage III Disease. Cancer research. 2004;64(21):7697.

144. Le Floc'h A, Jalil A, Vergnon I, Le Maux Chansac B, Lazar V, Bismuth G, et al. αEβ7 integrin interaction with E-cadherin promotes antitumor CTL activity by triggering lytic granule polarization and exocytosis. Journal of Experimental Medicine. 2007;204(3):559-70.